PURIFICATION OF AN EXTRACELLULAR β-GLUCOSIDASE FROM A STRAIN OF *Trichoderma reesei* CONTAINING MULTIPLE *bgl1* GENES

By

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To the ladies in my life,
Wei Yea and Emily, you give me
serenity in the midst of chaos.

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LIST OF ABBREVIATIONS

BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
BG	B-glucosidase
BSA	Bovine serum albumin
CBH	Cellobiohydrolase
CBL	Cellobiono-δ-1,5-lactone
EG	Endoglucanase
ELISA	Enzyme-linked immunosorbent assay
HIC	Hydrophobic interaction chromatography
ICBR	Interdisciplinary Center for Biotechnology Research
Ig	Immunoglobulin
NHS	N-hydroxysuccinimidyl
PEG	Polyethyleneglycol
PBS	Phosphate buffered saline
pNPG	*p*-Nitrophenyl-β-glucopyranoside
SDS	Sodium dodecyl sulfate
Tris	Hydroxymethyl amino methane
Tween	Polyoxyethylene sorbitan monolaurate

Abstract of Dissertation Presented to the Graduate School
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Chairperson: Dr. Ross D. Brown, Jr.
Major Department: Food Science and Human Nutrition

Cellulose, the most abundant renewable resource, is a potential source of fuel and food. Its utilization may require a better understanding of the cellulases, especially β-glucosidase, which forms glucose from cellooligosaccharides. Many cellulolytic organisms either produce little extracellular β-glucosidase or retain a significant fraction of it in a cell-bound state. Recently a strain of ***Trichoderma reesei*** with an increased gene dosage for β-glucosidase has been reported. This strain produces a level of extracellular β-glucosidase some 50-fold greater than the parent strain. The most effective method of converting cellulose to fermentable sugars has been enzymatic saccharification; hence a clear picture of the role of β-glucosidase and its properties is essential. Elucidating properties of the enzyme may minimize the problems that affect cellulose utilization.

Successive anion and cation exchange chromatographies were used to purify the enzyme. Evidence for homogeneity was provided by the increase in specific

activity and by SDS gel electrophoresis. Kinetic properties, pH optimum and stability were determined for the purified enzyme.

In order to produce antibodies for immuno-purification process, white egg-laying chickens were injected with a solution of β-glucosidase (0.3 mg/mL) mixed with an equal volume of Freund's complete adjuvant biweekly, at different sites into the pectoral muscle. Eggs laid by the immunized hens were collected and anti-β-glucosidase antibody was isolated and purified from egg yolk by a series of precipitations with polyethyleneglycol. Female Muscovy ducks were immunized as described above. One week after each injection blood was collected and pooled. Protein A affinity chromatography was used to purify the duck antibody. Purified β-glucosidase and duck whole serum were sent to Cocalico Biologicals, Inc., Reamstown, PA, for the production of rabbit anti-β-glucosidase and rabbit anti-duck serum globulin antibodies, respectively. Samples were emulsified in Freund's complete adjuvant and inoculated subcutaneously into New Zealand White X Lop Ear rabbits for three consecutive weeks. The rabbits were bled 2 weeks after the last injection and sera were stored at -20 °C. Protein-G affinity columns were used to purify the antibodies.

The values of Km and Vmax for β-glucosidase were determined to be 0.14 mM and 54 units/mg using p-nitrophenyl β-D-glucopyranoside as substrate. The enzyme is optimally active from pH 4.5 to 5.0 and loses its activity at lower pHs. The isoelectric point was found to be pH 8.4. A molecular mass of 76 kDa was determined by SDS gel electrophoresis. Inhibition constants for glucose and 1-deoxynojirimycin were 638 μM and 0.62 μM, respectively. ELISA and Western

blotting confirmed specificity and sensitivity of the different antibodies against β-glucosidase. Affinity purification of β-glucosidase by hydroxysuccinimidyl-agarose chromatography has shown for every 20 mg of antibody, 0.4 mg of β-glucosidase was bound. Elimination of contaminants and reduction of traditional purification steps were possible, achieving the same specific activity value (43.5 units/mg of protein) and Km (0.14 mM) for the β-glucosidase from the native strain, using *p*-nitrophenyl-β-glucopyranoside as substrate.

INTRODUCTION

Lignocellulosic biomass is a unique renewable resource. The global photosynthetic process produces an estimated 1.5 x 10^{11} tons of dry plant material annually, of which about 50% is cellulose. It represents solar energy converted to chemical energy via the most fundamental biochemical reaction on our planet, photosynthesis. Life on earth can only be sustained via cycling of matter, and cellulolytic enzymes are critical to maintain the global carbon cycle as they initiate the degradation of cellulose, the major individual biomolecule in nature. This plant polysaccharide is used as an energy source by a plethora of microorganisms that occupy a variety of ecological niches as reviewed by Kadam (1996). Biomass in the form of lignocellulosic residues provides a means of harnessing and storing solar energy; hence, it represents an important renewable energy and material resource.

Because cellulose is the world's most abundant renewable carbon source, researchers have focused great interest on cellulose hydrolysis to produce glucose, which can be used to produce fuels, food and chemicals. Many steps have been made in strain improvement, optimization of culture conditions and characterization of cellulolytic enzymes at the biochemical and genetic level. Cellulosic material can be hydrolyzed using acid or enzymes. Acid hydrolysis of cellulose requires a relatively high temperature and more by-products are formed than with enzymatic hydrolysis (Persson et al., 1991). Intensive efforts have been made to develop more efficient cellulolytic enzymes and to increase the productivity of the enzyme-producing microorganisms.

Several decades have passed since cellulase from the fungus ***Trichoderma reesei*** was first described by Reese et al. (1950). Since then, this cellulase complex has been shown to consist of at least three types of enzymes that together convert native crystalline cellulose to oligosaccharides and glucose (Gritzali and Brown, 1979; Reese and Mandels, 1984): Endo-1,4-β-D-glucanases (EG, EC 3.2.1.4), which cleave internal glucosidic bonds; 1,4-β-D-glucan cellobiohydrolases (CBH, EC 3.2.1.91), which cleave cellobiosyl units from the ends of cellulose chains; and β-D-glucosidase (β-glucosidase, EC 3.2.1.21), which cleaves cellooligosaccharides and cellobiose to produce glucose. Each enzyme is, by itself, essentially inactive toward crystalline cellulose.

For the individual cellulase components and, in particular, the extracellular β-glucosidase the respective roles in cellulose hydrolysis and in the regulation of the cellulase enzyme system are under investigation by several research groups. The biochemistry and synergy between the different cellulases of ***T. reesei*** have not been fully resolved in precise molecular detail. However, the genes encoding cellobiohydrolase I (*cbh1*) (Shoemaker et al., 1983), cellobiohydrolase II (*cbh2*) (Chen et al., 1987; Teeri et al., 1987), endoglucanase I (*egl1*) (Pentilla et al., 1986) endoglucanase II (*egl3*) (Saloheimo et al., 1986), and an extracellular β-glucosidase (*bgl1*) (Barnett et al., 1991) have been cloned and sequenced These results represent an opportunity to look in more detail at the cellulase enzyme system by manipulating each individual component at the genetic level. Barnett et al. (1991) have demonstrated that the transformation of the *bgl1* gene into the ***T. reesei*** genome in multiple copies can be used to generate strains with significantly increased extracellular β-glucosidase activity.

The rate limiting step in cellulose hydrolysis is the one catalyzed by β-glucosidase. Since β-glucosidase is inhibited by its end product, glucose, the substrate cellobiose may accumulate and, in turn, inhibit the cellulase complex. If the rate of cellobiose hydrolysis could be increased, cellulose would become a more practical raw material for glucose production. This process will require a thorough understanding of the biochemistry and molecular biology of the enzymes catalyzing the conversion of cellulose to glucose.

Methods for transformation and genetic manipulation have been employed in strains of ***Trichoderma reesei*** resulting in the overproduction of extracellular β-glucosidase (Barnett et al., 1991). Whether the properties of this extracellular β-glucosidase were in good agreement with those of the natural enzyme produced by the native strain needed to be addressed.

The first specific research objective was to purify, compare and characterize the kinetic properties of β-glucosidase from native and hyperproducing strains. Another objective was to devise an immunosorbent method for the preparation of highly enriched β-glucosidase for biomass saccharification, using chicken, duck and rabbit polyclonal antibodies. Since traditional methods of purification were long and laborious, a highly specific affinity chromatography should reduce the purification steps with the same results as the traditional process.

REVIEW OF THE LITERATURE

Cellulose is the most abundant renewable biopolymer on Earth. Cellulose has been used by man for centuries; however, its enormous potential as a renewable source of energy was recognized only after the cellulose-degrading enzymes or "cellulases" had been identified. During World War II, the US Army was alarmed at the rate of deterioration of cellulosic material, including clothing, tents and sand bags, in the South Pacific (Bhat and Bhat, 1997). As a result of finding an immediate solution to this problem a parent strain QM6a was isolated from a shelter remains and was identified as ***Trichoderma viride*** by laboratories set up by several organizations within the Army and later was recognized as the distinct species ***Trichoderma reesei***. The immediate success of the U. S. Quartermaster Corps research program led to further research on selection and characterization of hyper-cellulolytic ***T. reesei*** strains. These projects not only improved the production of cellulase by ***T. reesei***, but also quickly led to the recognition that cellulosic wastes may be converted to glucose, soluble sugars, alcohols, single cell protein and other industrially useful chemicals through the agency of cellulases (Coughlan, 1985; Mandels, 1985; Reese and Mandels, 1984).

The past fifty years have witnessed remarkable progress in (a) isolation of microorganisms producing cellulases (Reese and Maguire, 1971); (b) improving the yield of cellulases by mutation, and protoplast fusion (Brown et al., 1986); (c) purification and characterization of the cellulases components (Bhat et al., 1993; Chirico and Brown, 1987; Christakopoulos et al., 1994; Wood and McCrae, 1982); (d) understanding the mechanism of cellulose degradation (Eriksson, 1978; Wood et al., 1989); (e) cloning and expression of cellulase genes (Barnett et al., 1991; Béguin and Aubert, 1993; Cummings and Fowler, 1996; Fowler and Berka, 1991); (f) determination of the 3-D structures of cellulase components (Dominguez and Alzari, 1995; Rouvinen et al., 1990); (g) understanding structure-function relationships in cellulases (Claeyssens and Tomme, 1989) and (h) demonstrating the industrial potential of cellulases (Coughlan, 1985).

Occurrence of Microbial Cellulase Enzymes

Cellulolytic enzymes are produced by a wide variety of bacteria and fungi, aerobes and anaerobes, mesophiles and thermophiles (Coughlan and Ljungdahl, 1988); however, relatively few fungi and bacteria produce huge levels of extracellular cellulase capable of solubilizing crystalline cellulose extensively (Wood, 1985). So far, most of the studies have been on the cellulase system of aerobic fungi ***Trichoderma viride***, ***Trichoderma reesei, Trichoderma koningii, Penicillium pinophilum, Fusarium solani, Sporotrichum pulverulentum*** and ***Talaromyces emersonii*** (Wood, 1975; Eriksson, 1978; Gritzali and Brown, 1979; Ogawa and Toyama, 1972). Only recently, it has been recognized that other microorganisms such as thermophilic aerobic fungi (***Sporotrichum thermophile, Thermoascus aurantiacus, Chaetomium thermophile, Humicola insolens***), mesophilic anaerobic fungi (***Piromonas communis, Sphaeromonas communis***), mesophilic and thermophilic aerobic bacteria (***Celluvibrio. sp, Cellulomomas sp, Thermomonospora sp***), mesophilic and thermophilic anaerobic bacteria (***Acetivibrio cellulolyticus, Clostridium thermocellum, Bacteroides cellusolvens***), as well asactinomycetes (***Thermomonospora fusca***), also produce active cellulases (Bhat and Bhat, 1997). Among the above noted microorganisms, the cellulolytic thermophilic microorganisms are of particular interest, because of their ability to produce thermostable cellulases which are generally stable under a variety of severe conditions including highly acidic and alkaline pHs at temperatures up to 90 °C (Lamed and Bayer, 1988). Notable thermophilic cellulolytic microorganisms are ***Clostridium thermocellum, Thermomonospra fusca, Thermoascus auarantiacus,*** and ***Sporotrichum thermophile*** (Bhat et al., 1993; Bhat and Maheshwari, 1987; Sugden and Bhat, 1994). These microorganisms are also capable of fermenting a wide range of substrates with minimal risk of contamination by other pathogens (Lamed and Bayer, 1988). Because of these advantages, the thermophilic cellulolytic microorganisms have attracted considerable research interest in recent years.

Fungal Cellulase Systems

The best-characterized cellulase systems are those of the aerobic fungi such as ***Phanerochaete chrysoporium*** (Eriksson, 1978), ***Talaromyces emersonii*** (McHale and Coughlan, 1980), ***Trichoderma koningii*** (Halliwell and Vincent, 1981; Wood and McCrae 1982), and ***Trichoderma reesei*** (Coughlan and Ljungdahl, 1988; Gritzali and Brown, 1979). The cellulase systems of these fungi consist of endo-1,4-

β-D-glucanase (EC 3.2.1.4), exo-1,4-β-D-glucanase (CBH, EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). The cellulase components of ***Trichoderma reesei*** are shown in Table 1.

Generally, the endoglucanases randomly attack H_3PO_4-swollen cellulose, CM-cellulose and the amorphous regions of crystaline cellulose and release cello-oligosaccharides. The CBHs hydrolyze H_3PO_4-swollen cellulose and Avicel sequentially by removing the cellobiose units from the non-reducing end of the cellulose chain (Wood and Bhat, 1988). Using radio-labeled cello-oligosaccharides, the ***Trichoderma*** CBH I was found capable of releasing cellobiose from the reducing end of cellulose chains (Vrsanska and Biely, 1992). The endoglucanase and CBH I of ***Trichoderma*** act synergistically to effect the extensive hydrolysis of crystalline cellulose, while the β-glucosidase completes the hydrolysis by converting the resulting cellobiose to glucose (Figure 1). Most of the fungal cellulases are glycoproteins and exist in multiple forms (Coughlan and Ljungdahl, 1988). Four immunologically related CBHs from ***T. viride*** appeared to differ from one another in the composition of covalently attached neutral carbohydrates (Gum and Brown, 1976). In contrast, ***T. reesei*** produced immunologically distinct CBH I and II (Nummi et al., 1983).

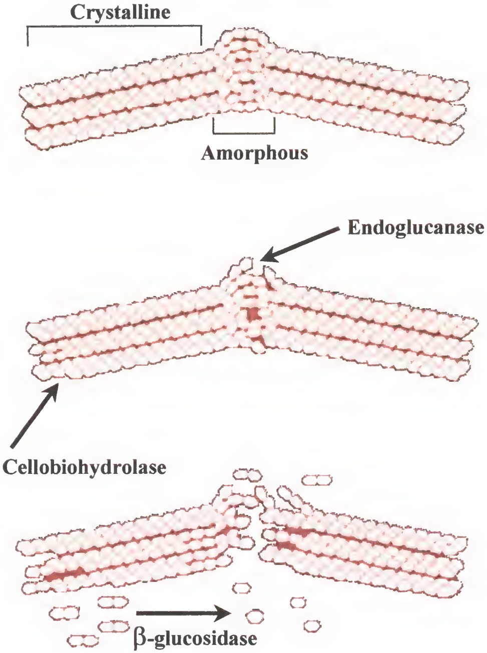
The extracellular cellulase components of most fungi are generally found to exist as individual entities (Coughlan, 1985). However, the presence of aggregates of extracellular enzyme in ***T. reesei*** culture filtrate has been reported; this complex consists of six proteins and exhibits cellulase, β-glucosidase and xylanase activities.

Table 1.
Enzymes comprising the cellulase system of ***Trichoderma reesei.***

Enzyme	MW (kDa)	pI
CBHI	60	4.2
CBHII	50	5.7
EG	49	4.7
EGII	35	5.5
EGIII	25	7.5
EGV	23	N/A
β-Glu I	75	8.5
β-Glu II	114	4.8

Source: Reinikainen, 1994.

Figure 1.
Cooperative action of enzymes of the cellulase system in hydrolytically converting cellulose to glucose.



Also, the proteins appear to be aggregated with the remnants of the fungal cell wall; Ca^{2+} may be involved (Sprey and Lambert, 1983). Although many fungi secrete separate cellulase components into the culture medium, it is not yet clearly known how these components interact on the surface of crystalline cellulose and their combined effect on the extensive hydrolysis of cellulose (Wood, 1992).

Measurement of Cellulase Activity

The physical heterogeneity of cellulosic substrates together with the complexity of cellulase systems produced by different microorganisms has led to the development of several assay procedures for the measurement of cellulase activities. The considerable difference in the nature of substrates used, variation in assay procedure adopted for measuring different cellulase components, and the synergistic action of cellulase components have made the comparison of results among laboratories difficult. In 1984, the IUPAC Commission on Biotechnology published a standard assay procedure for measuring cellulase activities (Ghose, 1984) introducing the filter paper unit of measurement (FPU/mL), where all assays for cellulase activity must be applied to an identical cellulosic substrate: Whatman No. 1 filter paper.

Some of these recommendations have been readily accepted by biotechnologists, but many enzymologists feel that these procedures are quite restricted and not satisfactory for understanding the mechanism of action and substrate specificities of cellulase in detail. Wood and Bhat (1988) reviewed the cellulase assays used by the laboratories working on fungal cellulases and highlighted the advantages and disadvantages of the various procedures. Although the procedures described were primarily for ***Trichoderma*** cellulases, those methods can be adapted for use with other cellulases. The substrates and the estimation methods used in measuring cellulase activities are summarized in Table 2.

Induction and Regulation of Cellulase Production

Cellulase is an inducible enzyme system (Kubicek, 1992; Ryu and Mandels 1980); all microorganisms studied so far have produced the highest level of cellulase when grown on cellulose (Wood, 1985). Cellobiose, lactose and sophorose are also known to facilitate the production of either a complete or an incomplete cellulase system by a few microorganisms (Mandels, 1960; Gritzali and Brown, 1979). Synthetic compounds such as palmitate and acetate esters of disaccharides and thiocellobiose have also been shown to function as inducers of cellulases. However, cellulose was found to be the best carbon source for the production of high levels of cellulase by many microorganisms (Ryu and Mandels, 1980; Wood, 1985). This observation raised a fundamental question with respect to induction and regulation of cellulase production, that is, how an insoluble substrate which cannot enter the microbial cell induces and regulates the production of cellulase.

The most generally accepted view of the induction process is that the low levels of cellulase constitutively produced by the microorganism first hydrolyzes cellulose to soluble sugars (Beguin and Aubert, 1993; Kubicek, 1992). These sugars are presumably converted into true inducers, which enter the cell and either directly or indirectly influence DNA binding protein and promote cellulase gene expression

Table 2.
Assay procedures and substrates used for cellulase activity measurements.

Enzyme	Substrate	Assay
Total cellulase	Cotton	-Estimation of cellulose residue -Reducing sugar released -Weight loss -Loss in tensile strength
	Filter paper, Hydrocellulose, Avicel and Dyed Avicel	-Release of dyed reducing sugars -Release of dyed soluble fragments
Exo-(1-4)-β-D-glucanase	Avicel	-Release of reducing sugars
	Hydrocellulose	-Release of dyed cellobiose
	Dyed Avicel	-Release of dyed cellobiose
	Amorphous cellulose	-Release of reducing sugar -Decrease in turbidity
	Substituted and unsubstituted cello-oligosaccharides	-Increase in reducing power -Analysis by HPLC
Endo-(1-4)-β-D-glucanase	Carboxymethyl Cellulose Hydroxyethycellulose	-Release of reducing sugar -Decrease in viscosity
	Substituted and unsubstituted cello-oligosaccharides	-Increase in reducing power -Analysis by HPLC
	Cotton	-Swelling in alkali
	Amorphous cellulose	-Release of reducing sugars
β-Glucosidase	Cellobiose	-Release of glucose
	Cello-oligosaccharides	-Increase in reducing power
	o- or p-Nitrophenyl-β-D-glucosides	-Release of o- or p-nitrophenol

Source: Wood and Bhat, 1988.

(Kubicek et al., 1993). It has also been suggested that in the case of ***Trichoderma***, the conidial-bound cellobiohydrolase hydrolyzes cellulose and releases cellobiose from which cellobiono-δ-1,5-lactone (CBL) may arise. The cellobiose and CBL are taken up by the mycelia and promote cellulase synthesis (Kubicek et al. 1993).

Mandels et al. (1962) predicted that cellobiose, the repeating unit of cellulose, could be the natural inducer of cellulase in ***T. reesei***. However, it has been shown with ***T. viride*** that cellobiose could induce cellulase production only at a high concentration (1% or more) or in the presence of a surfactant such as Tween 80 (Reese and Maguire, 1971). In contrast, cellobiose induced the production of endoglucanase by ***S. pulverulentum*** at a concentration of 1 mg/L (Ryu and Mandels, 1980). Also, Garcia-Martinez et al. (1980) reported that cellobiose induced CM-cellulase production at a concentration as low as 0.2%, and supported the production of the highest level of cellulase in the presence of 1% cellobiose in ***C. thermocellum***. Therefore, it can be argued that cellobiose is the natural inducer of the cellulase system, at least in some microorganisms.

It is generally accepted that the transglycosylation products from cellobiose could be the true inducer(s) of cellulase in ***T. reesei*** (Ryu and Mandels, 1980; Chirico, 1980). Also, it has been shown that sophorose (β-1,2-glucobiose), a contaminant in some glucose preparations, is an effective inducer of cellulase in ***T. reesei*** and in other species of ***Trichoderma***, even at a concentration of 0.3 μg/mL (Nisizawa et al., 1971). However, sophorose did not induce the production of cellulase in other fungi and in a mutant of ***T. reesei*** QM 9419. Thus, sophorose is not a universal inducer of cellulase. In contrast, Fritscher et al. (1990) reported that cellobiose induced the production of cellulase system in ***T. reesei***, either in the presence of β-glucosidase inhibitor or when a β-glucosidase defective mutant was used. These results strongly support the view that cellobiose could be the true inducer of the cellulase system in ***T. reesei***.

Studies using inhibitors of protein synthesis have suggested that cellulase formation is regulated at the translational level (Nummi et al., 1983). Evidence based on the measurement of mRNA levels documented that the regulation of the formation of cellulase occurs at the pre-translational level (Messner et al., 1991) and that cellulase gene transcription occurs within 20 min after the addition of inducer (El-Gogary et al., 1989). Except during conidiation, all cellulase inducers promoted the synthesis of a constant ratio of three major cellulases: CBH I, CBH II, and endoglucanase I in ***T. reesei*** (Kubicek et al., 1993). This indicated that the expression of all three major cellulase components is regulated coordinately. In contrast, investigation of the transcription of cellulase genes from other microorganisms revealed that transcription is not strictly coordinated. It has been found in ***C. thermocellum*** that transcription of several cellulase genes was induced sequentially when the cellobiose concentration in the medium became limiting (Béguin and Aubert, 1993). Hence, there is considerable variation in the level of transcription as well as the type of cellulase genes transcribed depending on the carbon source used for growth (Bhat and Bhat, 1997).

Carbon catabolite repression is another regulatory mechanism known to control cellulase production in bacteria and fungi. In this case, the end product of cellulose hydrolysis interacts with a cellular protein to form a complex which interacts with a particular gene at the transcription level and represses cellulase synthesis. Carbon catabolite repression occurs in ***E. coli***, ***C. thermocellum***, and ***S. cerevisiae*** (Bhat and Bhat, 1997). In the case of ***T. reesei***, growth on non-repressing substrates, slow feeding of glucose, or mutations which affect catabolite type repression did not induce cellulase synthesis to the same extent as in control experiments (Kubicek, 1992).

The evidence for carbon catabolite repression is based on the observations that no cellulase is formed during the growth of a microorganism on glucose, glycerol and other carbon source related to glycolytic metabolism. However, Messner at el. (1991) demonstrated that the addition of glucose to ***T. reesei*** cultures producing cellulases decreases the rate of formation of only CBH I to about 40%, while production of other cellulases continues up to 40 hrs. Because there is no clear evidence that either glucose or a catabolite in fact controls the transcription of cellulase genes, the term "catabolite repression" was recommended not to be used (Kubicek, 1992). The only alteration observed with these ***T. reesei*** mutants was at the level of the endoplasmic reticulum, suggesting that glucose may affect either the development of the secretory pathway or some of its components. In ***Trichoderma***, O-glycosylation of cellulases which takes place at the endoplasmic reticulum has been shown to control the secretion of cellulases. Because a decreased activity of dolichol-phosphate-mannose synthase, a key enzyme in the O-glycosylation pathway of ***T. reesei***, has been reported during growth on glucose (Kubicek, 1992), it is questionable whether glucose regulates the synthesis or the secretion of cellulases.

Although induction, catabolite repression, product inhibition and restricted carbon source supply are believed to be involved in the regulation mechanism of cellulase synthesis in fungi and bacteria (Merivuori et al., 1985), very little is known at the molecular level regarding the mechanism regulating the synthesis of cellulases. Recently, the presence of a putative regulatory protein in induced cells of ***Thermomonospora fusca***, and its binding to a 14-bp palindromic sequence located upstream from at least three endoglucanases genes have been reported (Béguin and Aubert, 1993). Therefore, further research at the molecular level on cellulase genes from different microorganisms is necessary for a better understanding of the regulation of cellulase synthesis.

Synergistic Interaction Among Cellulase Components

Synergism between cellulase components during the hydrolysis of cellulose was first demonstrated by Gilligan and Reese (cited by Bhat and Bhat, 1997). Subsequently, several researchers showed the cooperative action between endo- and exo-glucanases during the solubilization of crystalline cellulose and the release of various higher cellooligosaccharides and cellobiose (Wood and McCrae, 1972). β-glucosidase completes the hydrolysis by cleaving the cellooligosaccharides and cellobiose to glucose; however, the degree of synergism varied with the type of cellulose used. In the presence of highly ordered crystalline cellulose (cotton and Avicel) the synergism was high, with amorphous cellulose (H_3PO_4-swollen cellulose) it was low and it was absent in the presence of soluble derivatives such as CMC (Coughlan and Ljungdahl, 1988).

Four types of synergism have been reported between fungal cellulase components. These include: (a) endoglucanase and CBH (Wood and Bhat, 1988); (b) two immunologically related and distinct CBHs (Henrissat et al., 1985); (c) β-glucosidase and either endoglucanase or CBH (Wood et al., 1988). The fourth type involves a hypothetical non-hydrolytic protein called C_1 which may intervene in the relaxation of inter-molecular H-binding between adjacent cellulose chains. Although this hypothesis was never substantiated, isolation of CBH which acted synergistically with endoglucanase to degrade crystalline cellulose resolved this confusion (Wood and McCrae, 1979)

It has also been shown that CBH II tends to form complexes with endoglucanases, which act synergistically with the CBH I to degrade cellulose (Wood et al., 1989). Furthermore, CBHs I and II of ***P. pinophilum*** prepared free of endoglucanase act synergistically only to a limited extent during hydrolysis of crystalline cellulose. These results suggest that the exo/endo type synergism observed between CBH I and II of ***T. reesei*** could be due to the presence of trace amounts of endoglucanases (Wood et al., 1989).

Using highly purified endoglucanases and CBHs from ***P. pinophilum***, it has been demonstrated that specific types of endoglucanase must be added to the mixture of CBHs for the extensive hydrolysis of crystalline cellulose (Wood and Bhat, 1988). Thus, in the case of ***P. pinophilum*** cellulase, only two endoglucanases (EG III and EG V) which were strongly adsorbed onto cellulose showed a significant degree of synergism in presence of CBHs I and II (Bhat et al., 1990). Maximum synergistic activity occurred when the CBHs I and II were in 1:1 ratio, and with a trace amount of either endoglucanase III or V (Wood et al., 1988). These results from ***P. pinophilum*** demonstrated that at least three enzymes (two CBHs + one endoglucanase) are required for hydrolysis of crystalline cellulose, confirming the findings of Gritzali and Brown (1979) for ***Trichoderma***. Although the number and type of enzymes required to degrade crystalline cellulose have been established, it is still not well understood how the cellulase components interact on the face of the cellulose molecule (Bhat and Bhat, 1997).

β-Glucosidase

The β-(1-4) glycoside hydrolases, like all enzymes; are grouped and classified according to their specificities and action patterns. As a class of enzymes however, the β-glucosidases (EC 3.2.1.21) display very broad specificity with respect to both the aglycon and glycon moietes of substrates. For instance, natural substrates include the steroid β-glucosides and β-glucosylceramides of mammals, cyanogenic β-glucosides of plants secondary metabolism and the cellulosic plant cell walls (Clarke et al., 1993). A review of the literature indicates that almost all β-glucosidases have subunit molecular weights of 55 to 65 kDa and acidic pH optima (pH 5-6). These enzymes have attracted the attention of biochemists and bioorganic chemists alike in view of the simplicity, relative to other β-glycosidases, of both their mode of action and their substrates. These initiatives have greatly contributed to the understanding of the β-glycosidases and yet their mechanism of action remains a major topic of debate. Much of the mechanistic detail has been delineated from studies on the cellulolytic β-glucosidases of bacteria and fungi.

The microbial degradation of cellulosic biomass requires the action of at least three groups of enzymes: endo-glucanase, cellobiohydrolase and β-glucosidase which act synergistically to hydrolyze the β-1,4 bonds of cellulose to glucose (Henrissat et al., 1985). β-Glucosidase binds soluble cello-oligosaccharides in one productive mode and hydrolyzes glucosyl residues sequentially from the non-reducing end. The substrate-binding region of the ***Trichoderma reesei*** β-glucosidase I is composed of three to four subsites with the catalytic groups located between subsites one and two (Chirico and Brown, 1987). Kinetic studies with ***Aspergillus niger, Pyricularia oryzae*** and ***Sclerotium rolfsii*** enzymes suggest this is a common feature of all cellulolytic β-glucosidases (Clarke et al., 1993).

Homologous Amino Acid Sequences of Glycosyl Hydrolases

The complete or partial amino acid sequences are known for over 300 glycosyl hydrolases, of which approximately 85 are β-1,4-glucanases or xylanases (Clarke et al., 1993). Comparison and alignment of these sequences together with hydrophobic cluster analysis revealed considerable homology between fungal and bacterial enzymes and enabled the establishment of classification schemes comprising six (Henrissat et al., 1989), seven (Béguin, 1990) or eight (Gilkes et al., 1991) families. An analogous system was subsequently developed by Henrissat (1991) to include all the glycosyl hydrolases. Of the 35 families, ten involve the β-glucanases and xylanases with the β-glucosidases comprising families 1 and 3. Thus, with the paucity of detailed structural and mechanistic data, it is not surprising that predictions of catalytic residues and domains for the β-glucosidases were made based on homology studies with other well known β-glycanases such as hen egg white lysozyme (Clarke et al., 1993).

A highly conserved region has been observed among the aligned sequences of 16 different cellulases which contains an -Asn-Glu-Pro- motif. Site-directed mutagenesis of two of the genes encoding cellulases that display very little homology supported the prediction that the Glu residue was essential for activity (Baird et al., 1990). This motif was subsequently shown to exist in the Family 1 β-glucosidases (Gräbnitz et al., 1991) and the homologous region was extended to include conserved Arg and His residues. The roles of these latter residues were investigated by site-directed mutagenesis of the ***Clostidium cellulolyticum*** cellulase A gene (Belaich et al, 1992). The Arg residue is speculated to either participate in a salt bridge that maintains the structural organization of the enzyme or hydrogen bond to the substrate transition state. The His 122 residue was thought to be more essential for catalysis but it is unlikely to be the proton donor since substituting His 122 with Ser, Glu, Gly or Phe did not totally abolish catalytic activity. The His residue identified in the catalytic center of ***Clostidium thermocellum*** cellulase by chemical modification experiments likewise was concluded not to be the proton donor following site-directed mutagenesis studies (Tomme et al. 1991). On the other hand these authors suggested that the Glu of the -Asn-Glu-Pro- motif may serve as the proton donor during catalysis.

Mechanism of Catalysis of β-Glucosidase

Using site-directed mutagenesis on fungal and bacterial genes substantial progress has been made, and there is considerable evidence for the involvement of carboxylate ions in catalysis (Trimbur et al., 1993; Fowler, 1993b; Clarke et al., 1993). However, the active center of any β-glucosidase has not been entirely and unequivocally mapped. Similarly, any relation between the mechanism of catalysis and the essential specificity for a β-glycosyl moiety or the varying specificities for an aglycone or glycone group remains to be elucidated.

The mechanism of catalysis is one of the central questions that needs to be addressed for β-glucosidases. It is known that from studies of substrate specificity of β-glucosidases from a variety of sources, that there exist broad glycon and aglycon specificities. This is so whether they normally hydrolyze a specific natural substrate or not. Thus, the data and its interpretation regarding the mechanism of β-glucosidase catalysis have to be consistent with the observed broad glycon and aglycon specificities (Esen, 1993). Equally important are the mechanism of binding to substrate and the sequence in which binding between the glycone and aglycone portions of the substrate and the enzyme catalysis site occurs.

Clarke et al. (1993) reviewed the amino acid sequence similarity, kinetic, inhibition, chemical modification and affinity labelling, and mutagenesis data from studies on β-glucosidases mostly of fungal and bacterial origin. The data strongly support the involvement of acidic amino acids (Glu and /or Asp) as the general acid catalyst and as the stabilizing anion/nucleophile during catalysis. In fact, the involvement of the same amino acid residues in catalysis has been shown or proposed for all β-glycanases including β-glucanases, xylanases and lysozymes in addition to β-glucosidases (Esen, 1993). Clarke et al. (1993) concluded that β-glucosidases use a double displacement mechanism to hydrolyze their substrates. Two acidic amino acid residues (Asp/Glu) at the active center participate in the hydrolysis and there is a covalently linked enzyme-substrate intermediate. The basic tenets of this mechanism were first proposed by Koshland (cited by Clarke et al., 1993) almost 50 years ago and, as recently reviewed by Sinnott (1990), its revised form involves the following features:

1. an acid catalyst which protonates the substrate;
2. a carboxylate group of the enzyme positioned on the side of the sugar ring opposite the aglycone;
3. a covalent glycosyl-enzyme intermediate with this carboxylate in which the anomeric configuration of the sugar is opposite to that of the substrate;
4. this covalent intermediate may be approached from both directions through transition states involving oxocarbonium ions;
5. various noncovalent interactions providing most of the rate enhancement.

Identification of covalently linked enzyme-transition state intermediates has been used in understanding the mechanism of catalysis by a variety of enzymes. This approach can be used in combination with other promising and productive approaches such as site-directed mutagenesis targeting the specific amino acids forming the active site. Trimbur et al. (1993) have used both of these techniques in order to define the residues critical to catalysis as well as the mechanism of catalysis by a β-glucosidase of ***Agrobacterium sp***. Using tritiated 2-4-dinitrophenyl-2-deoxy-2-fluoroglucoside as a mechanism based inhibitor and peptide sequencing. The result showed that Glu 358 is the nucleophile which attacks the glycosidic bond of the substrate and to which glucose is covalently linked during catalysis. Site-directed mutagenesis studies targeting Glu 358 and other conserved (catalytically important) residues around Glu 358 showed clearly that it is the nucleophile responsible for stabilization of the transition state. For instance, each of nine different mutations at Glu 358 reduced the activity 10^4-fold or more with only the conservative substitution Asp giving measurable activity although it was 2500 fold lower. Drastic reductions in activity were also observed when Gly 360 and Asp 374 were mutated (Trimbur et al., 1993). These studies strongly indicated that the acidic residues are crucial to the catalytic action of ***Agrobacterium sp*** β-glucosidase; Glu 358 as the nucleophile in the formation of the covalent enzyme-glycosyl intermediate, and Asp 374 as the acid-base catalyst to protonate the leaving group (glucose) and then to deprotonate the water. Another conclusion drawn from these studies was that the substrate (aglycon) binding and catalytic sites are different and distinct because during enzyme inhibition by substrate analogues catalysis can continue.

Functions of β-Glucosidase

The role of cellulase gene products including β-glucosidase in cellulose hydrolysis and in regulation of the cellulase enzyme system has been under careful scrutiny for the past few years. Attempts have been made to obtain β-glucosidasedeficient strains of ***T. reesei*** by mutagenesis with either UV light (Mishra et al., 1989) or gamma irradiation (Strauss and Kubicek, 1990). However, the inability to obtain non-leaky β-glucosidase null mutations suggested that either β-glucosidase is an essential gene (involved in morphogenesis or as an essential structural component of the cell wall) or there are at least two different β-glucosidase genes in ***T. reesei*** (Chen et al., 1992). In 1999, Takashima et al. cloned the *bgl2* gene from ***T. reesei***. The deduced amino acid sequence was comprised of 466 amino acids, possessing a weak β-galactosidase activity in comparison to its homologue from the cellulolytic fungus ***Humicola grisea.***

Undoubtedly, one function of β-glucosidase is the breakdown of cellobiose produced by the cellobiohydrolases and endoglucanases to provide glucose as a carbon source for the microorganism. Expression of the cellulase genes is tightly regulated, being repressed in the present of glucose and induced in the presence of cellulose as a sole carbon source. However, since glucose results from the breakdown of a largely insoluble polymer substrate, a mechanism presumably exists to release soluble oligosaccharides from cellulose which can mediate the induction of the cellulase gene. A low constitutive level of cellulase expression has been postulated (El-Gogary et al., 1989) which could likely result in low levels of cellobiose in the presence of cellulose. Another function of the β-glucosidase may be to use the resultant cellobiose and glucose (via transglycosylation reaction) to produce oligosaccharides that have been shown to act as potent inducers of the cellulase enzyme system (Gritzali and Brown, 1979; Kubicek, 1987).

The deletion of the *bgl1* gene from ***T. reesei*** (Fowler and Brown, 1992; Fowler et al., 1993) provides a tool to study the role of extracellular β-glucosidase in the regulation of the other enzymes of the cellulase mixture and its function on the hydrolysis of cellulose. Results from experiments on the effect of the absence of the *bgl1* gene product on cell growth have indicated that the *bgl1* gene product is nonessential but its presence results in a more rapid induction of the cellulase enzyme system (Fowler and Brown, 1992). The observation that induction of the cellulase enzymes occurs, even if delayed in the absence of *bgl1* gene product, suggests that other processes are apparently capable of substituting for the proposed inducer formation function of β-glucosidase. However, this functional alternative occurs with a reduced efficiency resulting in a lag in production of cellulase mRNA and a more moderate decline in mRNA levels (Fowler and Brown, 1992). It is possible that other cellulase genes play a role in the mechanism that detect the presence of cellulose in the surrounding environment resulting in the efficient induction of the cellulase enzymes of ***T. reesei*** (Fowler, 1993b). For instance CBHII has been implicated as having a role in cellulase gene regulation (Messner et al., 1991; Seiboth et al., 1992). It remains to be seen to what extent the other cellulase enzymes are involved in the process of induction.

Genetic Manipulations of ***T. reesei*** β-Glucosidase I

Wild type strains of ***T. reesei*** produce extracellular cellulase at levels of a few grams per liter and in weight ratios of about 60:20:10:1 to CBH I:CBH II:EG I:BG I (Gritzali and Brown, 1979). In the biotechnology industry, selection of ***T.***

reesei production strains by mutagenesis and screening have resulted in strains capable of secreting cellulase enzymes (or components) in excess of 40 g/L (Durand et al., 1988). Alteration of cellulase component ratios was achieved by blending with other cellulase preparations (Enari et al. 1981; Kadam and Demain, 1989) or by changing the fermentation conditions. However, these methods tend to be expensive and/or labor intensive.

Since the mid 1980s, methods for transformation and genetic manipulation of ***T. reesei*** became possible with the introduction of both dominant and auxotrophic markers. Techniques such as electroporation and protoplast fusion for introducing the foreign DNA into the fungal cells (Pentilla et al., 1987; Gruber et al., 1990) have been employed to manipulate the cellulase enzymes of strains resulting in the complete removal or overproduction of either individual or multiple cellulase components (Barnett et al., 1991). This is very important as it means strains with novel cellulase profiles can be genetically tailored to defined applications. In addition, it has been possible to look more closely at the role of individual cellulase components in the biochemistry and synergy of cellulose hydrolysis as well as investigating how the cellulase genes are coordinately regulated upon growth of the fungus on cellulose (Fowler, 1993b).

Recently, the capabilities to increase expression of valuable proteins became possible by genetic engineering of filamentous fungi like ***T. reesei.*** As a general rule increasing the copy number of a fungal gene through transformation leads to increased expression of that gene (Fowler, 1993a). Examples of this phenomenon include glucose oxidase (Whittington et al., 1990) and glucoamylase (Fowler et al., 1990) expressed in ***A. niger***.

Barnett et al. (1991) took a similar approach in obtaining enhanced expression of extracellular β-glucosidase. Extra copies of a genomic clone of *bgl1* were introduced into the genome of ***T. reesei*** using the transformation vector called pSASβ-glu. Positive selection of ***T. reesei*** transformants containing extra copies of bglI was made using the ***A. nidulans*** amdS gene as a selectable marker (Pentilla et al., 1987). ***Trichoderma*** does not contain a functional equivalent of the amdS gene and is therefore unable to utilize acetamide as sole nitrogen source unless the amdS gene is firmly inherited during transformation. The cellulase products from these multicopy bglI gene transformants showed an increase in the rate of glucose release from Avicel. More detailed analysis of one transformant has shown 5-10 additional copies of the bglI gene were integrated into the genome, which gave rise to a 4.2-fold increase in bglI mRNA levels (Fowler, 1993a). Also, an increase in the rate of production of glucose from different substrates (cellobiose and phosphoric acid swollen cellulose) was observed. These data suggest that integration of additional copies of bglI in the genome of ***T. reesei*** leads to an increase in specific message levels and corresponding extracellular protein levels. Moreover, the cellulolytic activity of transformed strains of ***Trichoderma*** was specifically improved with the insertion of the bglI gene.

Production of Polyclonal Antisera for Affinity Purification of β-Glucosidase

Antibodies are serum immunoglobulins with binding specificity for particular antigens. Although antibodies can be identified in the serum of individuals that have been exposed to particular pathogens, the usual methods for eliciting antibodies involve immunization with purified or partially purified antigen preparations. Antigens used are most commonly proteins and peptides (Cooper and Paterson, 1997).

The first consideration is usually whether polyclonal or monoclonal antibodies are needed. Gritzali and Brown (1979) and Hofer et al. (1989) produced polyclonal and monoclonal antibodies against β-glucosidase, respectively. However, these studies were done to compare the reactivity of β-glucosidase with other ***Trichoderma*** β-glucosidases and not for the intent of enzyme purification. Polyclonal antibodies are particularly valuable for immunoprecipitation and immunoblotting, whereas monoclonal antibodies can have exquisite specificity and can be derived for almost any purpose. Choice of the species of animal to be used for immunization is based in part on whether antibodies of great specificity are needed (Cooper and Paterson, 1997).

The amount of antibody required must also be evaluated. Clones of hybridomas (somatic cell hybrids of B cells from an immunized animal's spleen and myeloma tumors permissive for the production of monoclonal immunoglobulins) provide an essentially limitless supply of a constant reagent. Nevertheless, the initial investment in producing a monoclonal antibody is quite large, whereas relatively large amounts of polyclonal antiserum can be obtained from a single individual such as a rabbit or chicken.

Production of good antisera depends in large part upon the quality, purity, and the amount of available antigen as well as on the specificity and sensitivity of the assay. For protein antigens, if possible, the material should be biochemically homogeneous and depending on the intended use may be either a native or denatured conformation. Minor contaminants are often (unfortunately) more antigenic than the immunogen of interest, and antisera resulting from immunization may have more activity against the contaminants than against the protein of interest.

Although the advances offered by the development of monoclonal antibody techniques have revolutionized the specificity, uniformity and quantity of antibodies, many circumstances remain in which polyclonal antibodies are more desirable than monoclonal antibodies (Cooper and Paterson, 1997). Production of polyclonal antisera takes less time and effort than production of monoclonal antibodies, requires relatively simple and readily available equipment, and produces reagents than can be used for immunoprecipitation, immunoblotting, and ELISAs.

Traditionally, rabbit has been used as the best source for specific antisera because of the ease of maintenance and high quantity of antibody concentration in whole blood (30 to 50 mL can be obtained at each bleed). The life span of a rabbit is 5 to 6 years, so a continual source of specific antiserum can be provided over a period of time by one rabbit after booster injections. Moreover, the recommended times between booster injections are not critical; the animal may be rested for several months between subsequent boosters after the primary and secondary booster injections. However, blood collection must take place 7 to 14 days after each booster immunization to ensure a high antibody titer (Harlow and Lane, 1988).

Avian species represent an alternative source for large-scale antibody production. The use of hen egg yolk as a source of specific antibody offers considerable advantages such as compatibility with modern animal protection regulations, low cost, and convenience. Moreover, because of the evolutionary distance of this immunoglobin from mammalian antibodies, IgY antibodies may be used in serological tests which are affected by rheumatoid factors (Hassl and Aspöck, 1988).

Chickens have long been used in the vaccine industry, so there are few regulatory impediments. Vaccine against flu and polio are widely adopted, and the few viral diseases transmissible from avians to humans are well described and controlled (Morrow, 2000). Transgenic farm chickens are now widely used in the production of human therapeutic proteins in which the potential for expression of complex proteins in their proper three-dimensional configuration with post-translational modifications exemplify one of the advantages.

Chicken transgenic antibodies are being considered as additives in toothpaste or mouthwashes in order to prevent periodontic disease. Such applications would require amounts greater than 10000 Kg/year, a substantial challenge for the present day processing technology. The cost of constructing a facility for housing chickens is approximately $1 M, quite modest compared to fermentation facilities for bacteria or mammalian cells. The cost of chicken eggs in commercial facilities is $0.05 per egg, and each egg yields approximately 25 mL of egg yolk of which 2.5 gr. is globulin (Morrow, 2000). Furthermore, a single rooster can provide 2000 inseminations per month and the time for maturation from egg to offspring is less than six months, by contrast, other species take much longer to generate. For all the advantages mentioned above, avian species (chicken and duck) were chosen in this study, to produce polyclonal anti-β-glucosidase antibodies and to compare the sensitivity and specificity of these immunoglobulins against a traditionally used mammal (rabbit) as source of antibodies.

MATERIALS AND METHODS

Purification of β-glucosidase

Culture filtrate of ***Trichoderma reesei*** containing multiple bgl1 genes (β-glu C5X), a generous gift from Genencor International (Palo Alto, CA), was dialyzed against 15 L of sodium succinate 50 mM, pH 6.0 buffer containing 3 mM NaN_3 (buffer A) and then concentrated to 500 mL. Purification procedures were followed as described by Chirico and Brown (1987).

An aliquot of 140 mL of the dialyzed culture filtrate solution was applied to a 14 x 36 cm DEAE-Sephadex A-50 (Pharmacia. Alameda, CA) anion-exchange column equilibrated with buffer A. Aryl-β-glucosidase activity was eluted isocratically at a flow rate of 400 mL/h and the effluent collected in 200 mL fractions. Cellobiohydrolase I and a negligible amount of aryl-β-glucosidase activity were eluted with 50 mM buffer A, pH 3.6, which contained 0.5 M NaCl and 3 mM NaN_3. Fractions enriched in aryl-β-D-glucosidase activity from DEAE-Sephadex A-50 column (buffer A) were combined, equilibrated and concentrated with 5 mM imidazole, pH 6.0 which contained 3 mM NaN_3 (buffer B) in a Amicon Diaflo Utrafilter (Amicon. Lexington, MA), the general retentivity was >10000 MW (Amicon PM10 ultrafiltration membrane). This material was applied to the same DEAE-Sephadex A-50 anion-exchange column equilibrated with buffer B. Aryl-β-D-glucosidase activity was eluted isocratically and collected in 200 mL fractions at a flow rate of 400 mL/h. Cellobiohydrolase II, endoglucanase activity and a negligible amount of aryl-β-D-glucosidase activity was eluted with 5 mM imidazole buffer, pH 6.0, which contained 3 mM NaN_3 and 0.5 mM NaCl.

Fractions eluted isocratically from the DEAE-Sephadex A-50 column (buffer B) enriched in aryl-β-D-glucosidase activity, were combined, equilibrated, and concentrated as mentioned above with 20 mM sodium succinate, pH 6.0 containing 3 mM NaN_3 (buffer C). A fraction (~35 mL) of this solution was applied to a 4.4 x 55 cm SP-Sephadex C-50 cation-exchange (Pharmacia. Alameda, CA) column equilibrated with buffer C. Aryl-β-D-glucosidase activity was eluted isocratically and collected in 6.2 mL fractions at a 60 mL/h flow rate. Fractions enriched in aryl-β-D-glucosidase activity were concentrated and stored until needed. A negligible amount of aryl-β-D-glucosidase activity subsequently was eluted from the column with buffer C, containing 0.5 M NaCl. The absorbance of each fraction was measured at 280 nm using a Beckman DU-8 Double Beam Spectophotometer (Beckman Instruments. Irvine, CA). All chromatographic manipulations were performed at 4 °C.

Enzyme Activity Determination

Aryl-β-D-glucosidase activity was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-β-glycopyranoside (Sigma. St. Louis, MO) after incubation with β-glucosidase. Samples were incubated for 20 min at 40 °C in 1.5 mL of 50 mM sodium acetate buffer, pH 5.0 containing 3 mM NaN_3 and 2.4 mM *p*-nitrophenyl-β-glucopyranoside, the reaction was stopped by immersing the incubation tubes in boiling water for 10 min. After cooling at ambient temperature, 0.75 mL of

7.5% (w/v) K_2HPO_4 was added and the absorbance of *p*-nitrophenol was measured at 400 nm. Specific activity was expressed as μmol *p*-nitrophenol released/min/mg of protein.

Protein Quantification and Purity Determination

Protein content was determined according to the method of Lowry et al. (1951), as described by Bailey (1967), using a Beckman DU-8 Double Beam Spectrophotometer. Bovine serum albumin (Pierce. Rockford, IL) was used as standard. Protein purity was examined using sodium dodecyl sulfate (British Drug House. Poole, England) polyacrylamide gel electrophoresis (SDS-PAGE) after reduction, according to the method of Laemmli (1970). Mini-slab gels (7cm x 8cm) at 1mm thickness, consisting of stacking gel, 4% acrylamide - 0.1% bisacrylamide (British Drug House. Poole, England) and separating gel, 7.5% acrylamide - 0.2% bisacrylamide. Proteins (7.5 μg/well) were loaded and electrophoresis carried out in a Protean™ II Slab Cell apparatus (Bio-Rad. Hercules, CA) at constant current (20 mA) in a buffer (pH 8.3) containing 25 mM Tris-HCl (Sigma. St. Louis, MO) and 0.19 M glycine for 45 min. The purity of the protein preparations was determined by comparing gels stained with a Commassie Blue R-250 (Eastman Kodak. Rochester, NY) solution.

β-glucosidase activity was detected in polyacrylamide gels by the procedure described by Lojda (1970). Gels were washed at room temperature for 30 min in 0.5 M sodium acetate buffer, pH 5.0, which contained 3 mM NaN_3. The buffer was replaced every 10 min with fresh buffer during the 30 min infusion. Gels were incubated in substrate solution at 40° C for 30-40 min until a green color developed and then stored in 7% (v/v) acetic acid. Substrate solution was prepared immediately prior to use by dissolving 3 mg of 5-bromo-3-chloro-3-indolyl-β-D-glucopyranoside (Sigma. St. Louis, MO) in 2 mL of methanol. To this mixture was added 5 mL of 50 mM sodium acetate buffer, pH 5.0, which contained 3 mM NaN_3, followed by the addition of distilled water to a final volume of 10 mL.

Isoelectric Focusing

Isoelectric focusing was performed in 5% (w/v) polyacrylamide gels as described by Garfin (1990). Purified β-glucosidase was loaded (5 μg/well) into a mini-slab gel (7cm x 8cm) at 1mm thickness and allowed to electro-focus at 100 volts for 1 h, 200 volts for 1 h, and 500 volts for 30 min. Standard proteins (Sigma. St. Louis, MO) used were: amyloglucosidase (pI 3.6), soybean trypsin inhibitor (pI 4.6), bovine β-lactoglobulin A (pI 5.1), bovine carbonic anhydrase II (pI 5.9), human carbonic anhydrase I (pI 6.6), myoglobin (pI 6.8, band 1), myoglobin (pI 7.2, band 2), lactate dehydrogenase (pI 8.3, isomer 1), lactate dehydrogenase (pI 8.4, isomer 2), lactate dehydrogenase (pI 8.6, isomer 3), and trypsinogen (pI 9.3). Gradients of pH were prepared using a pH 3-10 ampholyte solution (Sigma. St. Louis, MO).

Amino Acid Analysis

Purified enzyme was subjected to SDS-PAGE in a Tris-Tricine discontinuous buffer system (Schagger and von Jagow, 1987) and then electrophoretically blotted to polyvinylidene difluoride membranes and stained with Coomassie Blue R-250

(Plough et al., 1989). The band corresponding to β-glucosidase (5-10 μg protein) was excised and subjected to gas-phase acid hydrolysis (Ozols, 1990) followed by amino acid analysis as the phenylisothiocarbamate derivative using an Applied Biosystems 420A instrument (Foster City, CA). This analysis was performed by the Protein Chemistry Core facility of the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida.

Effect of pH on Enzyme Stability

Purified β-glucosidase was preincubated in 50 mM sodium acetate solutions containing 3 mM NaN_3, which had been adjusted to pH values ranging from 3.0 to 7.0. The effect of exposure to different hydrogen ion concentrations on stability was measured by the release of *p*-nitrophenol at pH 5.0, using *p*-nitrophenyl-β-glucopyranoside as substrate. The assays were conducted at 40 °C. The pH of each mixture was determined before and after incubation, the activity remaining was determined as the average of eight replicate incubations.

Determination of pH Optimum

The effect of hydrogen ion concentration on β-glucosidase activity was obtained using *p*-nitrophenyl-β-glucopyranoside as substrate. Assay mixtures were incubated for 20 min at 40 °C in 1.5 mL of 50 mM sodium acetate buffer, containing 3 mM NaN_3 and 2.4 mM of the substrate and then the pH was adjusted to different values with HCl or NaOH. To elucidate the effect of hydrogen ion on Vmax and Vmax/Km, Lineweaver-Burk plots were obtained at pH values from 3.0 to 7.0, using *p*-nitrophenyl-β-glucopyranoside as substrate at concentrations varying from 0.2 to 2.4 mM. The pH determined before and after incubation and was found not changed by more than ± 0.1 pH units. Each activity value was the average of eight replicate incubations.

Determination of Inhibition Constants

Inhibition by glucose or 1-deoxynojirimycin (Sigma. St. Louis, MO) of β-glucosidase was determined in the presence of *p*-nitrophenyl-β-D-glucopyranoside as substrate, at concentrations ranging from 0.2 to 2.4 mM, pH 5.0. Inhibition constants (Ki) were calculated from replots of Lineweaver-Burk and Dixon plots. The points on each Lineweaver-Burk plot represent the value calculated from the average of eight replicate incubations. Replots of Lineweaver-Burk were constructed by plotting the slope of each double reciprocal plot (at a fixed concentration of inhibitor) vs. the corresponding inhibitor concentration. The slope of each Dixon plot (at fixed concentration of substrate) was replotted vs. the corresponding reciprocal concentration of substrate. Kinetic constants (Vmax, Km, and Ki) were also calculated by nonlinear regression using EZ-FIT software (Perrella Scientific. Amherst, NH) for comparison.

Chicken Immunization and Antibody Purification

White egg-laying chickens were injected biweekly with a purified solution of β-glucosidase (0.3 mg/mL) incorporated into a same volume of Freund's complete adjuvant (Sigma. St. Louis, MO), at different sites into the pectoral muscle. Eggs laid by the immunized chickens were collected and anti-β-glucosidase antibodies were isolated and purified using the method of Polson et al. (1985) and a simplified procedure of Hassl and Aspöck (1988).

As described by Polson et al. (1985), one part egg yolk was added to four parts (v/v) 0.1 sodium phosphate buffer (pH 7.6). The mixture was made up to 3.5% (w/v) polyethyleneglycol (PEG 6000) and stirred for five min. Following centrifugation at 5000 X g (10 °C) for 20 min, the supernatant collected was made up with 8.5% (w/v) PEG. The suspension was allowed to stand for 10 min followed by centrifugation at 5000 X g (10 °C) for 25 min. The pellet was dissolved in 2.5 volumes (v/v) sodium phosphate buffer 0.1 M (pH 7.6) and was made up with PEG to 12% (v/v). Again, the suspension was allowed to stand for 10 min and then centrifuged at 5000 X g (10 °C) for 25 min. The pellet was resuspended in ¼ volume phosphate buffer and cooled to 0 °C before adding an equivalent volume of 50% ethanol (-20 °C). Following centrifugation at 10000 X g (4 °C) for 25 min, the precipitate was dissolved in ¼ volume phosphate buffer and the suspension was dialyzed overnight (4 °C) against 4 L 0.1 M sodium phosphate buffer (pH 7.6). After dialysis, the antibody preparation was made up with NaN_3 to 0.1% and stored at -20 °C until needed.

For the procedure by Hassl and Aspöck (1988), the immunuglobulins were extracted by hydrophobic interaction chromatography (HIC). One volume of yolk was thoroughly mixed with 4 volumes of 0.1 M sodium phosphate, containing 0.15 M NaCl, pH 7.0 (PBS) and 4.4% (w/v) PEG 6000, incubated for 30 min at room temperature, and then centrifuged at 4750 X g for 60 min. The aqueous supernatant was applied to a Phenyl-Sepharose CL-4B (Pharmacia. Alameda, CA) column (1.5 x

70 cm, bed volume = 75 mL) at a rate of 2 mg protein/mL of gel. For the removal of the unbound material the column was washed with diluted PBS (0.0116 M). Elution was performed with deionized water, the eluted protein was collected and frozen at -20 °C. After about 5 repetitions, the column was washed with 75% ethylene glycol in PBS 0.1 M, pH 7.0 to remove any strongly bound substances.

Duck Immunization and Antibody Purification

Female muscovy ducks were immunized as described previously. One week after each injection blood was collected by making a small incision underneath the wing and was pooled. Serum was separated by centrifugation at 5000 X g (4 °C) and subjected to PEG precipitation. One volume of serum was thoroughly mixed with 4 volumes of 0.1 M sodium phosphate, containing 0.15 M NaCl, pH 7.0 (PBS) and 4.4% (w/v) PEG 6000, incubated for 30 min at room temperature, and then centrifuged at 4750 X g for 60 min and stored at -20 °C.

Duck anti-β-glucosidase antibody was subjected to chromatography with a Protein A Sepharose CL-4B, cyanogen bromide activated (Sigma. St. Louis, MO) column (10 mm diameter x 100 mm length, Sigma. St. Louis, MO) as described by Higgins et al. (1995). The gel (approximately 5 mL when packed) was first washed extensively in deionized water, then equilibrated in 0.02 M sodium phosphate buffer, pH 7.0, washed with 0.1 M glycine-HCL, pH 3.0, then with 0.1 M tris (hydroxymethyl) aminomethane (Tris)-HCL (Sigma. ST. Louis, MO), pH 8.7, and finally again with sodium phosphate buffer. An aliquot of two mL of sera (49 mg/mL, pH 7.0) was applied to the column slowly and once within the gel flow was stopped for 30 min. The sample was then washed out with at least 200 mL sodium phosphate buffer (0.02 M, pH 7.0). Bound proteins were eluted at the fastest as possible flow rate (~2mL/min), with 0.5 M glycine-NaOH, pH 11.5; the effluent was monitored at 280 nm and was collected in one mL samples into tubes containing approximately 0.25 mL 4 M glycine-HCL, pH 5.0 to neutralize the pH. Samples within peaks were pooled and dialyzed overnight against 0.1 M PBS, pH 7.4, and then stored at -20 °C. The column was regenerated by extensive washing with cycles of 0.1 M Tris-HCL, pH 8.7, and 0.1 M glycine-HCL, pH 3.0, and was stored at 4 °C in sodium phosphate buffer (0.1 M, pH 7.6) containing 10% ethanol.

Rabbit Immunization and Antibody Purification

Purified β-glucosidase and duck whole serum were sent to Cocalico Biologicals, Inc. Reamstown, PA, for the production of rabbit anti-β-glucosidase and rabbit anti-duck serum protein antibodies respectively. Samples were emulsified in Freund's complete adjuvant and inoculated subcutaneously into New Zealand White X Lop Ear rabbits for three consecutive weeks. The rabbits were bled 2 weeks after the last injection and sera were stored at -20 °C. Frozen rabbit antisera were thawed and subjected to PEG precipitation. One volume of serum was thoroughly mixed with 4 volumes of 0.1 M sodium phosphate, containing 0.15 M NaCl, pH 7.0 (PBS) and 4.4% (w/v) PEG 6000, incubated for 30 min at room temperature, and then centrifuged at 4750 X g for 60 min and stored at -20 °C.

Protein-G affinity columns were used to purify the antibodies. From a HiTrap protein G affinity columns kit (Pharmacia. Alameda, CA), each column was prepared as described by the manufacturer. Columns were pre-equilibrated with 20 mL 0.1 M sodium-phosphate binding buffer. Two mL of sample were loaded to the columns which then was washed with 20 mL binding buffer. Proteins were eluted with 0.1 M glycine-HCl buffer, pH 2.8. Fractions of one mL were collected and pH was neutralized with 100 mg Tris crystals, stored at -20 °C until needed.

Molecular Weight Determination of Enzyme and Anti-β-glucosidase Antibodies

The molecular weight of the antibody preparation was determined using SDS-PAGE after reduction according to the method of Laemmli (1970). Mini-slab gels (7cm x 8cm) at one mm thickness, consisting of stacking gel (4% acrylamide - 0.1% bisacrylamide) and separating gel (7.5% acrylamide - 0.2% bisacrylamide) were prepared according to the Protean™ II Slab Cell Instruction Manual (Bio-Rad, 1985). Enzyme and antibody solutions were diluted with sample buffer containing β-mercaptoethanol (Sigma. St Louis, MO) and boiled for 5 min. After a ten µg aliquot was applied to each sample well, electrophoresis was carried out for 45 min at a constant current of 20 mA. An SDS-6H high molecular weight protein standard kit (Sigma. St. Louis, MO) containing carbonic anhydrase (29000), egg albumin (45000), bovine albumin (66000), phosphorylase (97400), β-galactosidase (116000), and myosin (205000) was used. Molecular weights of the antibody proteins were determined following the method of Weber et al. (1972).

Antibody Titer Determination by Enzyme-linked Immunosorbent Assay (ELISA)

One hundred µL of purified β-glucosidase containing 2.5 to 100 ng of protein in 0.1 M $NaHCO_3$, pH 8.6 (coating buffer) was applied to the sample wells of microplates (Costar. Cambridge, MA). Following overnight incubation at 4 °C, the well was aspirated and washed 4 times using the Nunc-Immuno Wash (A/S Nunc. Rochester, NY) with PBS-Tween (Sigma. St. Louis, MO) containing 0.1 M sodium phosphate, pH 7.2, 0.15 M NaCl, and 0.2% Tween 20. After 100 µL primary antibodies (chicken, rabbit, and duck anti-β-glucosidase antibodies) at amounts of 0.01 to 10 µg in PBS-Tween had been added to the wells, incubation was allowed to proceed at ambient temperature for 45 min. The aspirations and washing were repeated as described previously before 0.1 mL of secondary antibody (Sigma. St. Louis, MO), rabbit anti-chicken IgG-alkaline phosphate conjugate, and goat anti-rabbit IgG-alkaline phosphate conjugate were added to the wells containing chicken and rabbit anti-β-glucosidase antibodies respectively. Subsequent incubation, aspiration and washing were carried out as explained above, with the exception of wells containing duck anti-β-glucosidase, where ten ng of rabbit anti-duck antibody in 75 µL PBS-Tween was added, followed by 45 min of incubation at room temperature. The microplate was aspirated and washed again with PBS-Tween, followed by the addition of 0.1 mL of goat anti-rabbit IgG-alkaline phosphate conjugate and soon afterward incubation and washing were applied.

Assay buffer (0.05 M Na_2CO_3 and 0.05 M $NaHCO_3$ containing 0.0005 M $MgCl_2$) was added to the wells (75 µL), the plates were incubated at ambient temperature untill a yellow color developed (~15 min). The absorbance of the plates at 405 nm was monitored after 40 min of incubation using an ELISA reader (model 2550, Bio-Rad. Hercules, CA). Almond β-glucosidase (Sigma, St. Louis, MO), BSA (Pierce. Rockford, IL), as well as culture filtrates of ***Hypocrea schweinitzii*** and ***Aspergillus niger*** (Genencor International. Palo Alto, CA) were used to check for possible cross reactivity.

Immunoblotting

Purified β-glucosidase was subjected to SDS-PAGE under reducing conditions (Laemmli, 1970) and then electro-transferred to a nitrocellulose membrane (Bio-Rad. Hercules, CA). Mini-slab gels (7cm x 8cm) at one mm thickness, consisting of stacking gel (4% acrylamide - 0.1% bisacrylamide) and separating gel (7.5% acrylamide - 0.2% bisacrylamide), were prepared as described previously. A constant current of 18 mA/gel was applied during separation. Aliquots containing ten ng of β-glucosidase were applied to each well and run with the protein standards (SDS-6H Molecular Weight Marker Kit, Sigma. St. Louis, MO).

Following electrophoresis, the gels were equilibrated in 400 mL of Towbin transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol). Electro-transfer was performed according to the Trans-Blot Cell Instruction Manual (Bio-Rad, 1989) at a constant voltage of 50 V for 3 hrs using Towbin buffer as the electrolytic buffer. Complete transfer of protein was verified by staining the gels with Coomassie Blue solution.

Following electro-transferring of proteins, the nitrocellulose membranes were rinsed with PBS 0.01 M, pH 7.2 (0.01 M sodium phosphate, pH 7.2, containing 0.15

M NaCl and 0.2% w/v NaN_3), and incubated with Blot/Tween blocking solution (5% w/v nonfat dry milk, 0.2% v/v Tween 20, and 0.02% w/v NaN_3 in PBS) at room temperature with agitation for 1.5 hr. After washing twice for five min each in PBS, the membranes were incubated overnight in the primary antibody PBS solution (10 ng/mL). Following washing with four changes of PBS for five min each, the membranes were treated for one hr with rabbit anti-chicken IgG-alkaline phosphate conjugate and goat anti-rabbit IgG-alkaline phosphate conjugate as secondary antibody, for chicken and rabbit respectively. In the case of membrane containing duck anti-β-glucosidase antibody, the secondary rabbit anti-duck antibody was added (PBS, 10 ng/mL) and allowed to incubate at ambient temperature for three hrs. After incubation, the membrane was washed as described earlier goat anti-rabbit IgG-alkaline phosphate conjugate was added and allowed to incubate at room temperature. All membranes were then washed again with PBS as described, and incubated with 20 mL of 5-bromo-4-chloroindolyl phosphate/Nitro blue tetrazolium solution (BCIP/NBT, Sigma Fast™, Sigma. St. Louis, MO) until bands appeared. The reaction was stopped by rinsing the membranes with deionized water. Almond β-glucosidase, BSA, as well as culture filtrates of ***Hypocrea schweinitzii*** and ***Aspergillus niger*** (Genencor International. Palo Alto, CA) were used as negative controls.

N-hydroxysuccinimidyl Chloroformate Activated Agarose Affinity Chromatography

Protein coupling was performed as described by Cuatrecasas and Parickh (1972) with modifications detailed below. N-hydroxysuccinimide (NHS) cross-linked 4% beaded agarose (Sigma. St. Louis, MO) was mixed with an equal volume of ice-cold distilled water, removing the liquid immediately with gentle suction, repeating the washing step with cold water for three times. The resin was transferred to cold coupling buffer (100 mM $NaHCO_3$, pH 8.2) containing 10 to 100 mg/mL protein (anti-β-glucosidase antibody). The solution was mixed gently for 48 h at 4 °C and then was washed five times with three volumes each time of cold coupling buffer. The beads then were transferred to two volumes of 1 M ethanolamine (Sigma, St. Louis, MO), pH 8.5 (blocking buffer) and incubated at room temperature for 2 h with gentle agitation, followed by washing with distilled water until the eluant reached neutral pH. The gel was washed 10 times with two volumes each time of 1 M NaCl and ten times with two volumes each time of distilled water. The resin was packed into a 1 cm diameter x 10 cm length column (Sigma. St. Louis, MO) equilibrated in 0.02 M phosphate (Na_2HPO_4-NaH_2PO_4) buffer, pH 7.0, for immediate use or otherwise stored in 1 M NaCl containing 0.1 NaN_3 at 4 °C.

Enzyme bound to the antibody column was eluted at a flow rate of ~2 mL/min, with 1.0 M NH_4OH, pH 11.5, monitored at 280 nm and was collected in 0.5 mL fractions in tubes containing approximately 0.25 mL 4 M glycine-HCL, pH 5.0 to neutralize the pH. Samples within peaks were pooled, dialyzed overnight against 20 mM sodium succinate buffer, pH 6.0 containing 3 mM NaN_3 and then stored at 4 °C.

RESULTS AND DISCUSSION

Purification and Characterization of β-glucosidase

Purification and characterization of the components of ***Trichoderma reesei*** cellulase enzyme system have been reported (Gritzali and Brown, 1979; Chirico and Brown, 1987). Since a major part of the β-glucosidase activity remains bound to the cell wall (Messner and Kubicek, 1990), commercial preparations of cellulase are thought to be reduced in their ability to produce glucose because of the relatively low concentration of β-glucosidase. Improvement of the cellulase preparation by the addition of purified β-glucosidase (Enari et al., 1981; Kadam and Demain, 1989) or isolation of mutant strains of ***T. reesei*** are possible solutions to this problem.

The cloning and sequence analysis of an extracellular β-glucosidase gene bgl1 from ***T. reesei*** has been reported by Barnett et al. (1991). It has also been shown that transformation of the bgl1 gene into the ***T. reesei*** genome in multiple copies can be used to generate strains with significantly increased β-glucosidase activity (Barnett et al., 1991). In order to describe clearly the properties of this over produced β-glucosidase purification and characterization of the enzyme are required. Chirico and Brown (1987) described the isocratic elution of β-glucosidase from DEAE-Sephadex columns equilibrated with low ionic strength buffer at pH 6.0, where the endoglucanase and cellobiohydrolase II were retained. These procedures were utilized for the purification of the over produced β-glucosidase as described in Materials and Methods.

DEAE-Sephadex Chromatography (Succinate Buffer)

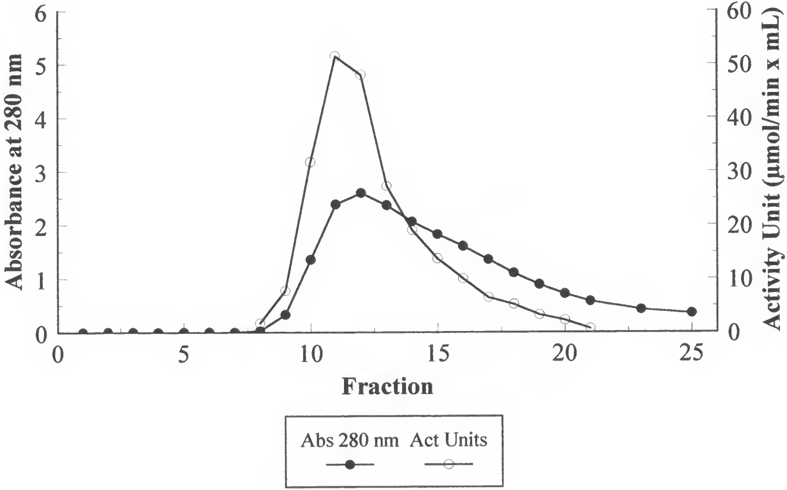
Culture filtrates of ***Trichoderma reesei*** (grown on a complex commercial medium) containing multiple bgl1 were prepared and applied to a DEAE-Sephadex A-50 anion exchange column as described in the Materials and Methods section. An aliquot of dialyzed culture filtrate (140 mL) that contained 5812 mg of protein and had a specific β-glucosidase activity of 12.5 units/mg was applied to an anion exchange column. The amount of protein in the starting material was determined using a modified method of Lowry as described by Bailey (1967). The profile obtained from the isocratic elution of this column is presented in Figure 2. Protein was eluted in one single peak and all β-glucosidase activity was located at the leading edge of the protein peak. The column then was eluted with the same buffer adjusted to pH 3.6 and the ionic strength increased by the addition of 0.5 M NaCl. Cellobiohydrolase I and a negligible amount of β-glucosidase activity were found in this elution, as reported by Chirico (1980). Fractions (10-13) enriched in β-glucosidase activity were combined. Electrophoretic patterns of proteins eluted during each step of purification are presented and discussed later in this section.

DEAE-Sephadex Chromatography (Imidazole Buffer)

Fractions enriched in β-glucosidase activity from the DEAE-Sephadex A-50 succinate buffer column were combined, dialyzed, concentrated and applied to

Figure 2.
Elution profile from DEAE-Sephadex column chromatography
(succinate buffer) of dialyzed culture filtrates of ***Trichoderma reesei***
containing multiple *bgl1* genes.

Each fraction contained 200 mL and was assayed using *p*-nitrophenyl-β-D- glucopyranoside as substrate.


Absorbance at 280 nm
0
1
2
3
4
5
6
Activity Unit (µmol/min x mL)
0
10
20
30
40
50
60
Fraction
0
5
10
15
20
25
Abs 280 nm
Act Units

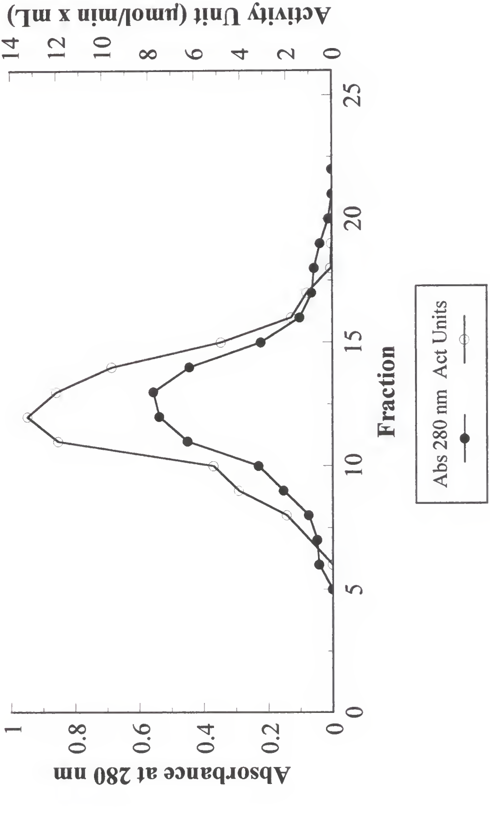
another DEAE-Sephadex A-50 anion exchange column, which had been equilibrated with 5 mM imidazole buffer as described in Materials and Methods. The sample applied to this column contained 1332 mg of protein (in 150 mL) and had a specific β-glucosidase activity of 23.4 units/mg. The elution profile from this column is shown in Figure 3. A single protein peak, which contained 80% of the β-glucosidase activity applied to this column, was eluted isocratically. When the ionic strength of the elution buffer was increased subsequently by the addition of 0.5 M NaCl, cellobiohydrolase II, endoglucanase I and a negligible amount of β-glucosidase were eluted from the column. Generally, all β-glucosidase activity is recovered during this step; however the amount of β-glucosidase in the sample applied to this column may have exceeded its solubility, which in pure water is approximately 1 mg/mL (Gritzali cited by Chirico, 1980). Fractions 9-15 containing β-glucosidase activity eluted from column I were combined.

The isocratic elution of β-glucosidase and the retention of cellobiohydrolase II and endoglucanase I by this column suggested that the β-glucosidase possesses a higher isoelctric point than these enzymes. Chirico (1980) determined the isoelectric points of cellobiohydrolase II and endoglucanase to be pH 5.6 and 4.7, respectively.

Alternatively, the β-glucosidase may be hydrophobic in nature and, due to its low surface charge density (Chirico, 1980), has little affinity for the anion exchange resin. Therefore, a sample of β-glucosidase was applied to a hydrophobic interaction column (Octyl-Sepharose) which retains hydrophobic proteins under conditions of high ionic strength. The isocratic elution from this column showed no increase in

Figure 3.
Elution profile from DEAE-Sephadex column chromatography (imidazole buffer) of combined fractions enriched in β-glucosidase activity eluted from the succinate buffer DEAE-Sephadex column.

Each fraction contained 200 mL and was assayed using *p*-nitrophenyl-β-D- glucopyranoside as substrate.


Absorbance at 280 nm
0
0.2
0.4
0.6
0.8
1
Fraction
0
5
10
15
20
25
Activity Unit (μmol/min x mL)
0
2
4
6
8
10
12
14
Abs 280 nm
Act Units

specific activity of β-glucosidase suggesting that most of the proteins eluted from the DEAE-Sephadex (imidazole buffer) column were not hydrophobic.

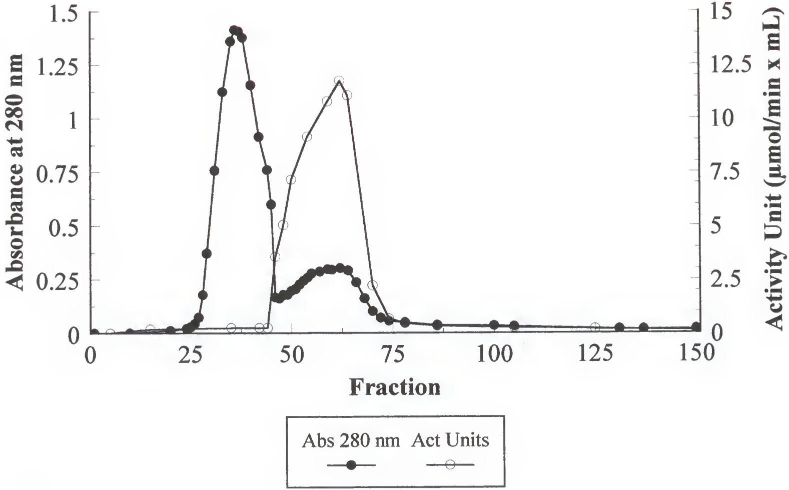
SP-Sephadex Chromatography

Combined fractions from the second DEAE-Sephadex A-50 imidazole column were dialyzed and applied to a SP-Sephadex C-50 cation exchange column, equilibrated with 20 mM succinate buffer. The sample applied to this column contained 178 mg of protein (in 60 mL) and had a specific β-glucosidase activity of 33.3 units/mg. The profile obtained during elution of this column is illustrated in Figure 4. Two peaks of material, which absorb light at 280 nm, and one peak of β-glucosidase activity were eluted isocratically. The total amount of β-glucosidase activity obtained in the elutate consisted of 91% of that applied to the column. The small amount of protein and β-glucosidase activity remaining on the column were eluted when the NaCl concentration of the elution buffer was raised to 0.5 M.

The isocratic elution profile suggested that one β-glucosidase is present in the sample. In contrast to the results reported by Chirico and Brown (1987), where three peaks of β-glucosidase activity were found and designated as peaks I, II, and III respectively. When Chirico and Brown (1987) stained the gels (representing the three peaks of activity) for carbohydrate, contaminating glycoproteins stained intensely, whereas the protein(s) responsible for β-glucosidase activity did not. These results suggested to the authors that complexes with the contaminants may have been responsible for the apparent multiples activity peaks and a further purification step of the β-glucosidase after SP-Sephadex C-50 chromatography using

Figure 4.
Elution profile from SP-Sephadex column chromatography
of combined fractions 10-13 eluted from the imidazole buffer
DEAE-Sephadex column.

Each fraction contained 6.2 mL and was assayed using *p*-nitrophenyl-β-D- glucopyranoside as substrate.


Absorbance at 280 nm
1.5
1.25
1
0.75
0.5
0.25
0
Activity Unit (µmol/min x mL)
15
12.5
10
7.5
5
2.5
0
0
25
50
75
100
125
150
Fraction
Abs 280 nm
Act Units

concanavalin A chromatography as described by Gong et al. (1979) might remove the contaminants. However, when β-glucosidase from the mutant strain was applied to a concanavalin A column, no increase in specific activity was observed (data not shown). This result indicated that the β-glucosidase and the minor contaminants contained little, if any carbohydrate.

Also, size-exclusion chromatography (Sephacryl 200) was used to attempt further purification of β-glucosidase obtained from the SP-Sephadex column. But neither complete removal of contaminating proteins nor increase in specific β-glucosidase activity was observed (data not shown). A summary of the purification steps and corresponding specific activity is shown in Table 3.

Electrophoresis Evidence for the Purity of β-glucosidase

Polyacrylamide gel electrophoresis evidence for homogeneity of the β-glucosidase purified by the methods described above is presented in Figure 5. A 7.5 μg sample of the enzyme yielded a single band when stained for protein with Coomassie Blue (lane 4) after successive chromatographies. A single band in the same location was also obtained for this protein when gel was stained for β-glucosidase activity. These indicate that the electrophoretically homogeneous protein is a β-glucosidase as well as a single monomeric polypeptide since electrophoresis was conducted under denaturing conditions.

To determine the molecular weight, β-galactosidase (130000), phosphorylase A (100000), bovine serum albumin (68000), ovalbumin (43000) and lactate

Table 3.
Purification summary of β-glucosidase.

Treatment	Protein (mg)	Total Activity (units)[a]	Specific Activity (units/mg)	Yield (%)	Purification Fold
Culture Filtrate	5812	72716	12.5 (0.2)[b]	100	1.0
DEAE Sephadex A-50 Succinate 50 mM	1332	31169	23.4 (3.7)[b]	43	1.9
DEAE Sephadex A-50 Imidazole 5 mM	178	5817	32.7 (14.0)[b]	8	2.6
SP-Sephadex C-50 Succinate 20mM	98	4363	44.5 (45.0)[b]	6	3.6

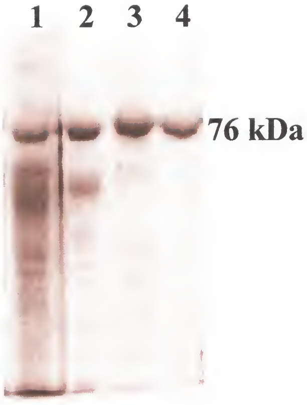
[a] Units are expressed as μmoles of PNP released/min.

[b] β-glucosidase specific activity for the native strain of ***T. reesei*** obtained by Chirico & Brown, 1987.

These results represent the average obtained from 1, 2, and 2 replications of step 1, 2, and 3, respectively.

Figure 5.
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 7.5% gel) profile of purified β-glucosidase from dialyzed culture filtrate of ***Trichoderma reesei***.

The gel represents from left to right, electrophoretic protein patterns stained with Coomassie Blue of culture filtrate (1), sample applied to DEAE-Sephadex imidazole buffer column (2), sample applied to SP-Sephadex column (3) and purified β-glucosidase eluted from the SP-Sephadex column (4).
Each sample well was loaded with 7.5 μg of protein.


1
2
3
4
76 kDa

dehydrogenase (36000) were used as molecular weight standards. A straight line was obtained when the molecular weight of each protein was plotted versus its mobility on a semi-logarithmic scale (data not shown). The relative mobility of the purified β-glucosidase yielded an estimated molecular weight of 76000. This molecular weight agrees fairly well with those calculated by Chirico and Brown (1987) and from its amino acid composition (Table 4).

The purified β-glucosidase differs in molecular weight from the β-glucosidase purified from culture filtrates of ***Trichoderma viride*** by Berghem and Pettersson (1974), reporting a molecular weight of 47000 kDa. The β-glucosidase described in this report has a lower molecular weight that of than the intracellular β-glucosidase isolated form ***Trichoderma reesei*** QM 9123 by Inglin et al. (1980), which reportedly has a molecular weight of 98000. The molecular weights of cellobiohydrolase I, cellobiohydrolase II and endoglucanase I are 53220, 54680 and 45215, respectively (Gritzali and Brown, 1979).

Isoelectric Point of β-glucosidase

To attain additional evidence for homogeneity and to determine the isoelectric point of the purified β-glucosidase, polyacrylamide gel electrofocusing was performed as described in Materials and Methods. The pH gradient extended from 3 to 10 and a single protein band was obtained when gels were stained with Coomassie Blue. This provided further evidence for homogeneity and demonstrated that the β-glucosidase is isoelectric at pH 8.4.

The isoelectric point of the β-glucosidase indicates that it is a basic protein and clearly distinguishes it from extracellular β-glucosidase purified from ***T. viride*** and from the intracellular β-glucosidase partially purified from ***T. reesei*** QM 9123, which have isoelectric points at pH 5.7 and 4.4, respectively (Chirico and Brown, 1987). The purified β-glucosidase is the most basic enzyme among the components of the cellulase system; cellobiohydrolase I, cellobiohydrolase II and endoglucanases reported in the literature have isoelectric points at pH 4.2, 5.7 and <7.6, respectively.

Composition of β-glucosidase

The amino acid composition of the purified β-glucosidase is presented in Table 4 and indicates a molecular weight of 78645 kDa. This enzyme is rich in aspartic acid, glycine, alanine, serine and basic amino acids and has few cysteine and methionine residues. The amino acid compositions of this β-glucosidase and the β-glucosidase purified by Berghem and Pettersson (1974) from ***Trichoderma viride*** when expressed as mole percent of constituent amino acids are similar. In all but four amino acids, these enzymes differ by less than 0.5 mole percent. The β-glucosidase described in this report contains a higher percentage of serine, valine and tryptophan and a lower percentage of proline. In contrast to cellobiohydrolase I, cellobiohydrolase II and the endoglucanase I purified from ***Trichoderma reesei*** by Gritzali and Brown (1979), the β-glucosidase contains significantly higher percentages of basic amino acids and a lower percentage of cystine residues.

The β-glucosidase purified by Chirico and Brown (1987) contains 0.70% by weight of total neutral carbohydrate using D-mannose as standard. Two residues of

Table 4.
Amino acid composition of β-glucosidase.

Amino Acid	Moles/Mole Protein*	Residue/ Protein Molecule**
Alanine	74	80
Arginine	23	29
Asparagine + Aspartic Acid	93	89 (43 Asp)
Cystine	6	7
Glutamine + Glutamic Acid	51	47 (15 Glu)
Glycine	78	77
Histidine	9	9
Isoleucine	7	20
Leucine	53	51
Lysine	25	25
Methionine	7	7
Phenyalanine	19	20
Proline	37	40
Serine	71	66
Threonine	51	55
Tyrosine	25	20
Tryptophan	22	15
Valine	67	64

Molecular Weight	79699 kDa* 75341 kDa**
Molecular Weight by SDS-PAGE	76000 kDa 74600 ± 2400 kDa*

*Chirico & Brown, 1987.
**Barnett et al., 1991 (inferred from *bgl1*sequence).

glucosamine, presumably as N-acetyl-glucosamine, were present in the enzyme suggesting that some oligosaccharides may have been linked to asparagine residues through N-glycosidic bonds. This carbohydrate composition clearly distinguished this enzyme from the β-glucosidase purified from ***Trichoderma viride*,** which has a neutral carbohydrate content of 10.3% by weight (Chirico, 1980). However, Berghem and Pettersson (1974) reported that the β-glucosidase from ***Trichoderma viride*** contains no detectable carbohydrate.

The β-glucosidase is one of the least glycosylated enzymes of those purified form extracellular filtrates of ***Trichoderma reesei***. Cellobiohydrolase I, cellobiohydrolase II and the endoglucanase I contain 5.04, 21.23 and 14.70% by weight of neutral carbohydrate, respectively (Gritzali and Brown, 1979). The lack of affinity for Concanavalin A suggests that the purified β-glucosidase of this investigation did not contain α-D-mannopyranosyl and/or α-D-glucopyranosyl terminal groups or internal 2-*O*-D-mannopyranosyl residues.

Relationship of pH to Enzyme Stability

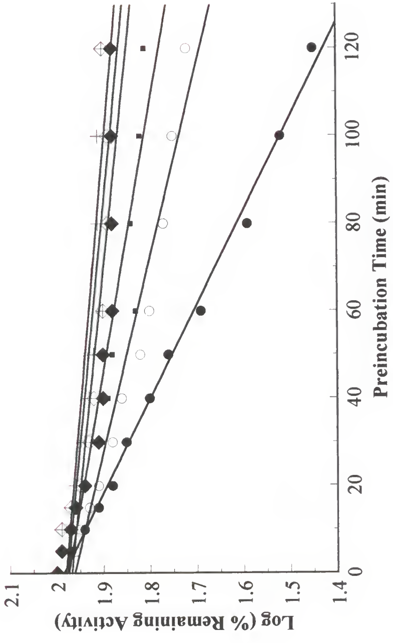
The effect of hydrogen ion concentration on stability of the purified β-glucosidase as determined by enzyme activity is illustrated in Figure 6. In sodium acetate buffer, pH 5.0, the enzyme is stable throughout the preincubation experiencing only 8% loss of activity after 120 min. However, preincubation at pH 3.5 and 4.0 results in a rapid loss of activity within 10 min followed by a gradual reduction to a residual level of activity at which 56% and 63% of original activity remains after 120 min, respectively. A similar rapid loss of activity within 10 min occurs at pH 3.0, although in this case, activity continues to decline reaching 27% of the original activity after 120 min. The purified β-glucosidase undergoes no significant loss of activity after 20 min preincubation in sodium acetate solutions having pH values from 5.0 to 7.0.

There are several possible explanations for the lability of the enzyme to acid conditions. Upon exposure to high hydrogen ion concentrations, the β-glucosidase may adopt a new conformation, which, is less active and more stable at low pH. Denaturation, as evidenced by loss of activity occurs at the lowest pH tested (pH 3.0) throughout the 120 min of preincubation. Gritzali and Brown (1979), suggested that loss of activity of the depolymerizing enzymes of ***Trichoderma reesei*** QM 9414 under alkaline conditions may be due to elimination of oligosaccharides linked through *O*-glycosidic bonds to serine or threonine. Since *O*-glycosidic bond linkages between oligosaccharides and proteins are stable under the acidic conditions of preincubation, a similar explanation with respect to the β-glucosidase is probably not valid. The high isoelectric point for this β-glucosidase (pI 8.4) indicates that repulsion of positively charged side chains accompanies the denaturation process.

The activity of β-glucosidase in filtrates of ***Trichoderma reesei*** QM 9414 grown on cellulose were observed to be low because of acid conditions which developed in the medium during cellulase production. Extracellular β-glucosidase activity increased when the medium had been buffered to pH 5.0. (Sternberg, 1976). The lability of purified β-glucosidase described in this report

Figure 6.
Effect of hydrogen ion concentration on the stability of purified β-glucosidase.

Purified β-glucosidase was preincubated in sodium acetate buffers at pH 3.0 (•), 3.5 (o),
4.0 (■), 5.0 (◇), 6.0 (+) and 7.0 (◆) and then assayed at pH 5.0 using
p-nitrophenyl-β-D-glucopyranoside as substrate.
The result represents the average of eight replicates.


Log (% Remaining Activity)
2.1
2
1.9
1.8
1.7
1.6
1.5
1.4
0
20
40
60
80
100
120
Preincubation Time (min)

at low pH values suggests that loss of β-glucosidase activity in filtrates of ***Trichoderma*** may result from irreversible denaturation of this enzyme.

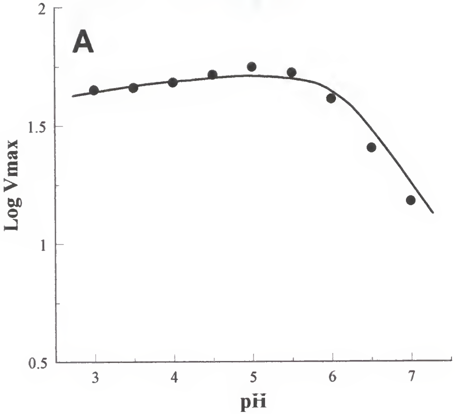
Optimum pH for Enzyme Activity

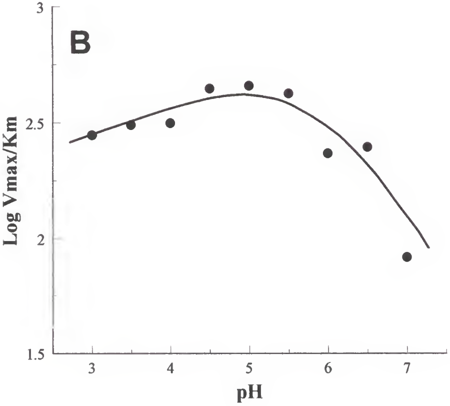
The effect of hydrogen ion concentration on enzyme hydrolysis and binding of *p*-nitrophenyl-β-D-glucopyranoside to purified β-glucosidase in sodium acetate solutions is shown in Figure 7. Dixon-Webb plots of log Vmax versus pH and log V/Km versus pH illustrate the effect of hydrogen ion concentration on catalysis. Kinetic constants were determined at each pH from Lineweaver-Burk plots using substrate concentrations from 0.21 to 2.40 mM. Assays were conducted at 40 °C for 20 min as described in Materials and Methods.

The rate of hydrolysis of *p*-nitrophenyl-β-glucopyranoside remains virtually constant and maximal from pH 3.0 to 5.0 (Figure 7A). Above pH 5.0, the rate of hydrolysis decreases indicating that the protonated form of an ionizable group, which has a pKa of 5.5, is necessary for catalysis. The pKa was estimated from the horizontal coordinate at the intersection of a horizontal line for Vmax and a line tangent to the plot at high pH as described by Segel (1976). Although the imidazolium ion of histidine has a range of pKa values from 5.5-7.0, the pKa of 5.5 obtained for the β-glucosidase is not sufficient evidence for the participation of an imidazolium ion in catalysis. In order to determine the identity of ionizable groups involved in catalysis, experiments such as active site labelling are needed. The line tangent to the plot at high pH has a slope of 0.54 indicating that the dependence of rate on pH may not be completely dominated by the ionization of a single group. In

Figure 7.
Dixon-Webb log plots: Log Vmax vs. pH and log Vmax/Km vs. pH.

Values for Vmax and Km were obtained from Lineweaver-Burk plots using *p*-nitrophenyl-β-D-glucopyranoside as substrate.
(A) Log Vmax vs. pH.
The intersection (pKa) was obtained by best fit lines which included points from pH 3.0 to 5.0 and from pH 6.0 to 7.0.
(B) Log Vmax/Km vs. pH.
The intersection (pKa) was obtained by best fit lines which included points from pH 3.0 to 5.0 and from pH 5.5 to 7.0.


A
Log Vmax
2
1.5
1
0.5
3
4
5
6
7
pH


B
Log Vmax/Km
3
2.5
2
1.5
3
4
5
6
7
pH

contrast, lines having slopes 1 or 2 indicate that the dependence of rate on pH is completely governed by one or two deprotonations, respectively (Tipton and Dixon, 1979).

The irreversible and complete denaturation below pH 3.0 prevents similar analysis at low pH. However, an upper limit of pH 3.0 may be established for the pKa of any group, which must be deprotonated in order to participate in catalysis. This upper limit suggests that a carboxylate anion of either glutamic or aspartic acid residue could be involved in catalysis (Chirico, 1980).

The pH variance of Vmax/Km is illustrated in Figure 7B. Vmax/Km represents the apparent first order rate constant for reaction of enzyme with substrate at low substrate levels; it reflects the ability of enzyme and substrate to form a complex. The apparent first order rate constant of the purified β-glucosidase remains essentially constant from pH 3.0 to 5.0. Above pH 5.0, Vmax/Km decreases indicating that the protonated form of an ionizable group, which has a pKa of 5.5, may be involved in stabilizing the form of the enzyme which combines with substrate. The tangent line to the plot at high pH has a slope of 0.53 indicating that the dependence of Vmax/Km on pH may not be completely dominated by the ionization of a single group. The similarities between the log Vmax and log Vmax/Km versus pH curves suggest that the same ionizable group may participate in both processes. Similar log Vmax and log Vmax/Km profiles have been obtained using β-galactosidase from ***E. coli*** (Chirico, 1980).

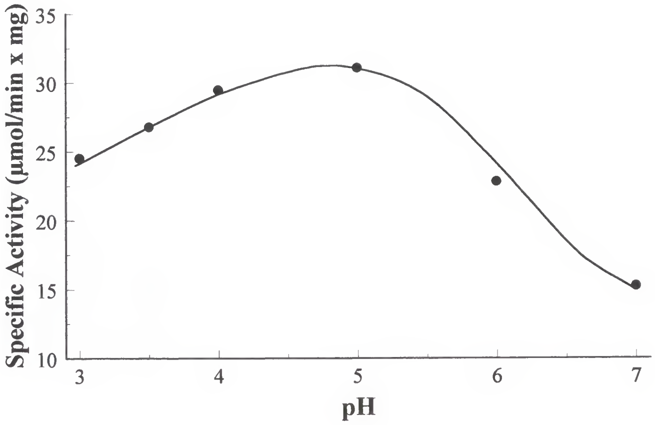
The dependence of the rate of β-glucosidase hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside on hydrogen ion concentration described in this report is different from that of extracellular β-glucosidase from ***Trichoderma viride*** (Maguire, 1977). When plotting Vmax versus pH, a sigmoidal dependence of hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside on pH was observed. The rate of hydrolysis increases from pH 4.5 to 2.0 and established an upper limit of 2 for the pKa of this group. Nevertheless, the rate of hydrolysis of the purified β-glucosidase described in this report remains virtually constant from pH 5.0 to 3.0. Determination of whether an ionizable group having a pKa <3.0 is also involved in catalysis or only a single group having a pKa of 5.5 is involved in catalysis is prevented by the denaturation of the β-glucosidase below pH 3.0.

The decrease in the rate of β-glucosidase hydrolysis above pH 5.0 described in this report is similar to that of the β-glucosidase from ***Trichoderma viride***; however, the pKa values estimated for the protonated group involved in catalysis differ. Maguire (1977) estimated the pKa of this group to be 7.3, where in this report the pKa was estimated to be at 5.5. Extracellular β-glucosidase isolated during the present investigation is distinguished from the intracellular β-glucosidase from ***Trichoderma reesei*** for which the pH optimum was found to be 6.5 and the lability at hydrogen ion concentration slightly removed from the optimum (Inglin et al., 1980).

The dependence of the rate of β-glucosidase-catalyzed hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside on hydrogen ion concentration is shown in Figure 8. The rate of hydrolysis remains essentially constant below pH 5.0 and decreases at higher pH values. Similar results were obtained when cellobiose and 4-methyl-umbelliferyl-β-D-glucopyranoside were used as substrates (Chirico and Brown,

Figure 8.
Optimum pH of purified β-glucosidase.

p-nitrophenyl-β-D-glucopyranoside (2.4 mM) was used as substrate.
The result represents the average of eight replicates.


Specific Activity (µmol/min x mg)
35
30
25
20
15
10
3
4
5
6
7
pH

1987). These results suggest that the mechanism of β-glucosidase-catalyzed hydrolysis for each substrate is similar.

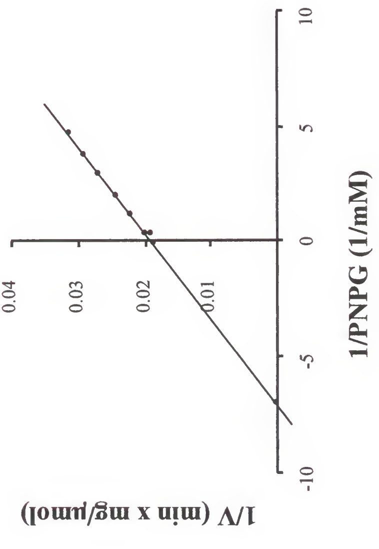
Determination of Kinetics and Inhibition Constants for the Purified β-Glucosidase

The specificity and mechanism of the purified β-glucosidase were investigated using *p*-nitrophenyl-β-D-glucopyranoside as substrate, and D-glucose and 1-deoxynojirimycin as inhibitors. Kinetic constants (Km and Vmax) were determined for the substrate using a least-squares analysis of Lineweaver-Burk plots (Figure 9) and EZ-FIT software (Perrella Scientific. Amherst, NH). Inhibition constants (Ki) were determined in the presence of substrate in separate experiments and calculated from replots of Lineweaver-Burk and Dixon plots, as well as by EZ-FIT software. Replots of Lineweaver-Burk plots were constructed by plotting the slope of each double reciprocal plot (at a fixed concentration of inhibitor) versus the corresponding inhibitor concentration. The slope of each Dixon plot (at a fixed concentration of substrate) was replotted versus the corresponding reciprocal concentration of substrate. A straight line through the origin of a Dixon replot demonstrates purely competitive inhibition (Segel, 1976).

Purified β-glucosidase was assayed at 40 °C in 50 mM sodium acetate buffer, pH 5.0, containing the appropriate substrate as described in Materials and Methods. The substrate concentrations used to determine kinetic and inhibition constants for *p*-nitrophenyl-β-D-glucopyranoside were from 0.21 to 2.40 mM. The concentrations of inhibitors used in these experiments are found in the figure legends.

Figure 9.
Lineweaver-Burk double reciprocal plot for purified β-glucosidase.

The reaction mixture contained 0.09 µg of purified β-glucosidase with *p*-nitrophenyl-β-D-glucopyranoside in 50 mM sodium acetate buffer, pH 5.0.
Substrate levels were 0.21, 0.26, 0.32, 0.48, 0.80, 2.00 and 2.40 mM.
The result represents the average of eight replicates.


1/V (min x mg/µmol)
0.04
0.03
0.02
0.01
-10
-5
0
5
10
1/PNPG (1/mM)

The values of Km and Vmax, as well as inhibitor constants, are presented in Table 5. For *p*-nitrophenyl-β-D-glucopyranoside, Km and Vmax were determined to be 0.14 ± 0.01 μM and 54.1 ± 0.82 units/mg. These values represent the average of those obtained from Lineweaver-Burk plots of the substrate in the absence of inhibitor. Chirico and Brown (1987) reported the affinity of the β-glucosidase for 4-methylumbelliferyl-β-D-pyranoside is five-fold greater than *p*-nitrophenyl-β-D-glucopyranoside and 60 fold greater than cellobiose. Furthermore, β-glucosidase catalyzes the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside at a rate greater than two-fold that of cellobiose. These results indicated that aryl-β-D-glucosides are preferred substrates for the purified β-glucosidase.

The Km value for *p*-nitrophenyl-β-D-glucopyranoside using the purified β-glucosidase described in this report is lower than the reported by Berghem and Pettersson (1974) using the extracellular β-glucosidase from ***Trichoderma viride*** in which the Km value was 0.28 mM. The value of Vmax for *p*-nitrophenyl-β-D-glucopyranoside determined for the β-glucosidase described in this report (54.1 units/mg) is similar to the Vmax (50.6 units/mg) determined using β-glucosidase from ***Trichoderma viride*** (Maguire, 1977).

The inhibitory effect of D-glucose on the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside catalyzed by the purified β-glucosidase is shown in Figure 10. The common intercept found for the Lineweaver-Burk plot at different inhibitor concentrations and the interception of Dixon plot at a point above the abscissa (Figure 11) indicate that D-glucose is a competitive inhibitor. A straight line

Figure 10.
Lineweaver-Burk plot of β-glucosidase inhibition by D-glucose using *p*-nitrophenyl-β-D-glucopyranoside as substrate.

Reaction mixture contained 0.09 μg of purified β-glucosidase with *p*-nitrophenyl-β-D-glucopyranoside in 50 mM sodium acetate buffer at pH 5.0. The fixed levels of inhibitor were 0 (■), 0.1 (▲), 2.0 (•) and 5.0 (◆) mM. The results represent the average of eight replicates.

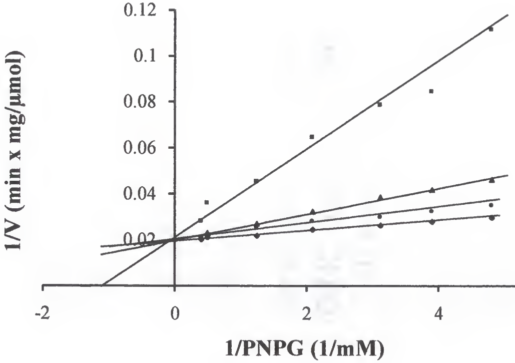
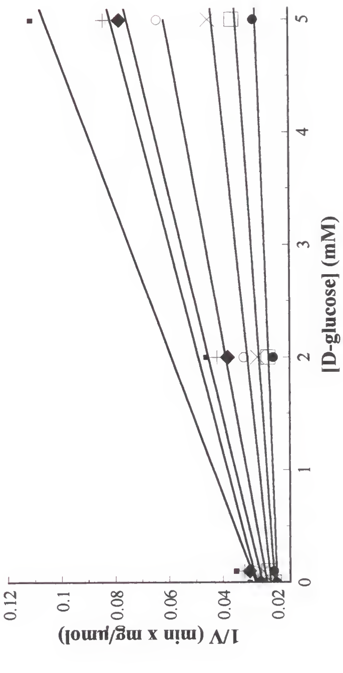

0.12
0.1
0.08
0.06
0.04
0.02
1/V (min x mg/µmol)
-2
0
2
4
6
1/PNPG (1/mM)

Figure 11.
Dixon plot of β-glucosidase inhibition by D-glucose using
p-nitrophenyl-β-D-glucopyranoside as substrate.

Substrate levels were 0.21 (■), 0.26 (+), 0.32 (◆), 0.48 (o), 0.80 (x), 2.00 (□) and 2.40 (•) mM.


1/V (min x mg/μmol)
0.12
0.1
0.08
0.06
0.04
0.02
0
1
2
3
4
5
[D-glucose] (mM)

through the origin indicating purely competitive inhibition was obtained when Dixon replot was constructed from the data presented in Figures 10 and 11 (Figure 14A). The inhibition constant for D-glucose obtained from Lineweaver-Burk and Dixon replot using *p*-nitrophenyl-β-D-glucopyranoside as substrate was 638 μM (Table 5).

The β-glucosidase purified during the investigation has an unusually high affinity for D-glucose, a product of hydrolysis. The extracellular β-glucosidase of ***Trichoderma viride*** also has a high affinity for this inhibitor (Chirico, 1980), although the affinity is less than that of the β-glucosidase isolated during the present investigation. Interestingly, the value of Ki for D-glucose using the purified β-glucosidase is approximately 2-fold less than the Km of cellobiose (Chirico and Brown, 1987). Assuming that Km is a good approximation of Ks, then the affinity of the enzyme for the product is 2-fold greater than the substrate. The high affinity of the purified β-glucosidase for D-glucose may serve as a sensitive point of regulation for the cellulase system (via transglycosylation reaction) to produce oligosaccharides that have been shown to act as potent inducers of the cellulase enzyme system (Gritzali and Brown, 1979; Kubicek, 1987).

The inhibition pattern obtained for 1-deoxynojirimycin during the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside is presented in the Lineweaver-Burk plot (Figure 12) and Dixon plot (Figure 13) and indicates that 1-deoxynojirimycin is a competitive inhibitor. A straight line through the origin of Dixon replot indicated purely competitive inhibition (Figure 14B). The inhibition constant obtained from Lineweaver-Burk and Dixon replot for this inhibitor using *p*-nitrophenyl-β-D-glucopyranoside as substrate is 0.61 μM (Table 5).

Figure 12.
Lineweaver-Burk plot of β-glucosidase inhibition by 1-deoxynojirimycin using *p*-nitrophenyl-β-D-glucopyranoside as substrate.

Reaction mixture contained 0.09 µg of purified β-glucosidase with *p*-nitrophenyl-β-D-glucopyranoside in 50mM sodium acetate buffer at pH 5.0. The fixed levels of inhibitor were 0 (■), 0.1 (▲), 2.0 (•) and 5.0 (◆) µM. The results represent the average of eight replicates.

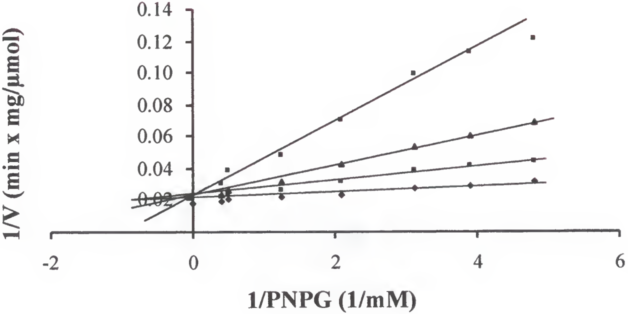

0.14
0.12
0.10
0.08
0.06
0.04
0.02
-2
0
2
4
6
1/V (min x mg/µmol)
1/PNPG (1/mM)

Figure 13.
Dixon plot of β-glucosidase inhibition by 1-deoxynojirimycin using
p-nitrophenyl-β-D-glucopyranoside as substrate.

Substrate levels were 0.21 (■), 0.26 (+), 0.32 (◆), 0.48 (o), 0.80 (x), 2.00 (□) and 2.40 (•) mM.

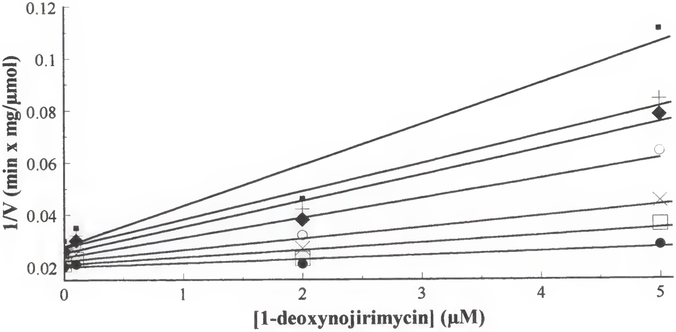

1/V (min x mg/µmol)
0.12
0.1
0.08
0.06
0.04
0.02
0
1
2
3
4
5
[1-deoxynojirimycin] (µM)

Table 5.
Kinetic and inhibition constants of β-glucosidase.

Substrate	Km (mM)	Vmax (units/mg)
p-Nitrophenyl-ß-D-glucopyranoside	0.14 ± 0.01[a]	54.1 ± 0.82[a]
	0.13 ± 0.03[b]	52.7 ± 0.61[b]
	0.10 ± 0.01[c]	56.9 ± 0.60[c]

	Ki (μM)	
	D-glucose	1-Deoxynojirimycin
p-Nitrophenyl-ß-D-glucopyranoside	638[d]	0.61[d]
	624[b]	0.59[b]
	570[c]	0.51[c]

[a] Values obtained from Lineweaver-Burk plot
[b] Values obtained by EZ-FIT software
[c] Native Strain (Chirico & Brown, 1987)
[d] Values obtained from Dixon replot

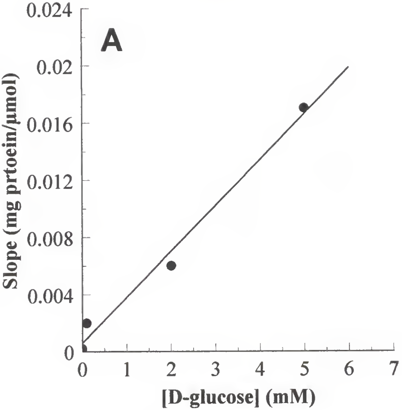
1-Deoxynojirimycin (5-amino-1,5-dideoxy-D-glucopyranose) is obtained by catalytic reduction of nojirimycin by hydrogen over PtO_2 or by $NaBH_4$. Niwa et al. (1970) reported that 1-deoxynojirimycin is a poor inhibitor of β-glucosidase from apricot emulsin, requiring a concentration of 25 mM to reach a 50% inhibition. However, many reports have demonstrated that 1-deoxynojirimycin is a powerful inhibitor of ***Trichoderma*** β-glucosidases (Chirico, 1980).

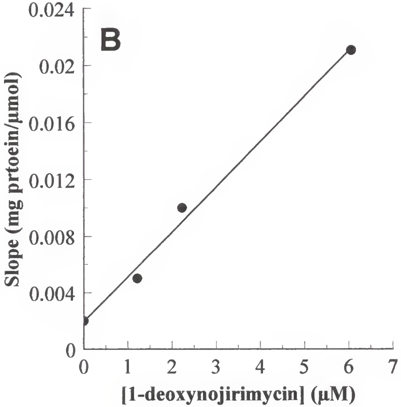
The weak inhibitory effect of 1-deoxynojirimycin on β–glucosidase from apricot emulsin was attributed to its lack of resemblance to the glycosyl cation (a partial positive charge is not present on C-1). Since 1-deoxynojirimycin does not assume a half-chair conformation in solution, it does not sterically resemble the glucopyranosyl cation. Yet this inhibitor is a more potent inhibitor of the β-glucosidase of ***Trichoderma*** than is D-glucono-1,5-lactone (Chirico, 1980). The pKa of 1-deoxynojirimycin is 6.6; under the conditions of assay (pH 5.0) this inhibitor is positively charged. A common feature of 1-deoxynojirimycin and the glucopyranosyl cation intermediate is the presence of a positive charge at or adjacent to carbon-1 of the ring. The positive charge is dispersed between C-1 and the pyranose ring oxygen atom in the glucopyranosyl cation, while in 1-deoxynojirimycin the positive charge is localized on the piperidine ring nitrogen atom (Chirico, 1980).

The enzyme-bound D-glucopyranosyl cation intermediate is stabilized by a negatively charged group in the active site; for instance, the carboxylate anion of an aspartic or glutamic acid residue (Clarke et al., 1993). Evidence supporting their

Figure 14.
Dixon replot of inhibition of β-glucosidase by D-glucose and 1-deoxynojirimycin.

(A) Inhibition by D-glucose and (B) Inhibition by 1-deoxynojirimycin, using *p*-nitrophenyl-β-D-glucopyranoside as substrate.


A
Slope (mg prtoein/µmol)
0.024
0.02
0.016
0.012
0.008
0.004
0
0 1 2 3 4 5 6 7
[D-glucose] (mM)


B
Slope (mg prtoein/µmol)
0.024
0.02
0.016
0.012
0.008
0.004
0
0 1 2 3 4 5 6 7
[1-deoxynojirimycin] (µM)

involvement in the mechanism of glycosidases comes from several sources (Chirico, 1980; Esen, 1993; Fowler, 1993b; Trimbur et al., 1993).

The disparity in the affinity of the β-glucosidases from apricot emulsin and ***Trichoderma*** for 1-deoxynojirimycin may reflect the proximity of the carboxylic acid group to the positively charged glucopyranosyl intermediate and inhibitors. In the former enzyme, the carboxylic acid group may be directly aligned with C-1 or positioned between C-1 and C-2, thereby unable to interact with the positive charge on the piperidine ring nitrogen atom of 1-deoxynojirimycin. However, the carboxylic acid group in the β-glucosidase from ***Trichoderma reesei*** and ***viride*** may be positioned on the opposite side of the sugar ring to the aglycone, thereby stabilizing the positively charged character of the inhibitor (Sinnott, 1990).

Production of Antibodies, Immobilization and Affinity Purification of β-glucosidase

In order to remove the minor contaminant bands and to determine the optimum purification method for β-glucosidase as well as high sensitivity of enzyme detection, production of antibodies from different animal sources was performed. The choice of animal for the production of antibodies depends upon the amount of antiserum desired, the evolutionary distance between the species from which the protein of interest has been derived and the species of the animal to be immunized. The use of chicken as an alternative source of polyclonal antibodies has been described by Polson et al. (1985) and adapted by many others (Gassmann et al., 1990). Like mammals, chickens protect their offspring by transferring maternal antibodies from serum to egg yolk (Rose et al., 1974). The properties of the 7S Ig species from avians are slightly different from those of mammalian IgG, and are called IgY (Warr et al., 1995). The quantitative advantage of raising IgY has been reported by Gottstein and Hemmeler (cited by Gassmann et al., 1990), where the amount of purified IgY produced in one month is 18 times higher than that of IgG produced in rabbit. Another advantage is that antigenic proteins are usually more immunogenic in the phylogenetically distant birds.

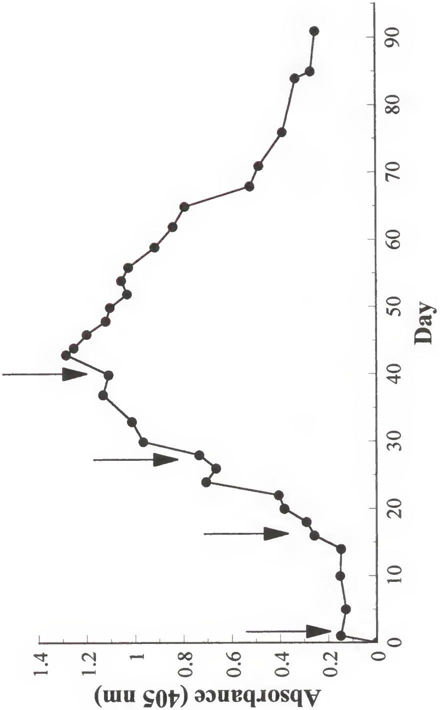
Duck antibodies have the same quality as chicken, however little has been done to utilize this animal as a source of antibody. Many studies have shown that the immune system of this animal is much more efficient in resistance to skin and other diseases that normally affect other avian species (Magor et al., 1992).

Chicken Antibody Production and Purification

Chickens were immunized with purified β-glucosidase as described in Materials and Methods to raise antibodies against the enzyme. In the presence of adjuvant, purified β-glucosidase was injected intramuscularly into an animal of the chosen species. Booster immunizations are started 2 weeks after the initial priming immunization and continue at 2-week intervals, while eggs were collected daily and stored. The profile of anti-β-glucosidase antibody production in an immunized hen is shown in Figure 15. The production of β-glucosidase-specific antibody did not occur until after the second boosting; peak activity occurred on day 44. Antibody activity decreased gradually with time following the fourth boosting. Eggs with the

Figure 15.
Profile of anti-β-glucosidase antibody production in egg yolks from immunized hens during the immunization period.

Purified β-glucosidase (0.3 mg/mL) used as antigen was injected biweekly into the animal.
↓ Indicates immunization boost.

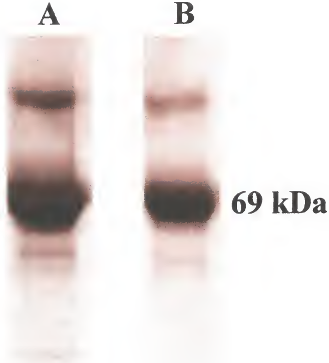

Absorbance (405 nm)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Day
0
10
20
30
40
50
60
70
80
90

highest activities were combined (days 40 to 49) for further purification of the antibodies by two different methods as described in Materials and Methods.

In the present report, two isolation procedures were used to purify chicken antibody. The purities of the antibody fractions were evaluated by SDS-PAGE gel as shown in Figure 16. Three distinct bands were observed, the molecular weight of the center band was estimated as 69 kDa which was close to the value reported for the heavy chain of IgY (Gassmann et al., 1990). Hassl and Aspöck (1988) used a combination of PEG precipitation and HIC to purify egg yolk immunoglobulins. At the PEG step of purification the protein content of 1 mL yolk mass was lowered from 60 mg to 41 mg and in the second step HIC reduced this amount to 5.4 mg. A photometric scanning study demonstrated that IgY comprised more than 85% of the total proteins (Hassl and Aspöck, 1988). Thus, the average yields of IgY antibodies obtained from eggs with 10 mL yolk were about 46 mg. In comparison, about 6.7 mg IgY antibodies was isolated from 1 mL initial yolk mass using the procedure described by Polson et al. (1985) As shown in Figure 16, no significant differences were detected between the purities of the IgY obtained by both methods. Although the Hassl and Aspöck (1988) method yields yolk antibodies more rapidly, the yield is lower than the two precipitation steps with PEG and alcohol respectively (Polson et al., 1985), this may not be a serious disadvantage since large quantities of yolks are usually available for processing.

Figure 16.
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 7.5% gel) profile of chicken anti-β-glucosidase antibody purified either by PEG precipitation or Phenyl Sepharose CL-4 B chromatography column.

Chicken anti-β-glucosidase antibody purified by PEG precipitation (A) and chicken anti-β-glucosidase antibody purified by Phenyl Sepharose column chromatography (B). Each well contained 10 μg of protein.


A
B
69 kDa

Duck Antibody Production and Purification

Female muscovy ducks were immunized with purified β-glucosidase as described in Materials and Methods to raise antibodies against the enzyme. The initial intention was to collect eggs from immunized animals and proceed to purify the anti-β-glucosidase antibodies. Unfortunately, due to the time of the year when the experiment was carried out, no eggs were laid by the animals, therefore a much more invasive procedure was performed on the ducks by collecting serum as a source of antibodies as described in Materials and Methods.

Extracts of bacteria, notably staphylococcal protein A and streptococcal protein G, have (by non-immune binding) contributed to the purification of the immunoglobulins (Igs) of many animals. Although the binding sites for protein A are poorly understood. Histidine appears to be an important contact residue, often in association with threonine (Higgins et al., 1995). Protein A chromatography, therefore, was considered as a useful tool for the purification of duck IgY with a His rich region, since it can rapidly yield substantial quantities of purified immunoglobulins. Higgins et al. (1995) evaluated the purification of duck immunoglobulins using protein A and protein G affinity chromatography. The result indicated that IgY bound to protein A efficiently and to protein G weakly. Hence for the present investigation protein A affinity chromatography was used to purify duck antibodies.

Proteins of duck serum gave substantial elution peaks from protein A at pH 11.5, by analysis of protein content 92% of total IgY was recovered. After five successive chromatographies a total of 109 mg of antibodies was obtained. To assess the level of non-Ig contaminants and the efficiency with which IgY was purified, the pH 11.5 eluate was examined in SDS-PAGE (Figure 17). A purified major band was observed with minor contaminants, notably albumin (Figure 17-2); the molecular weight was consistent with ducks larger IgY (MW 180 kDa, heavy chains 67 kDa) (Warr et al., 1995).

Rabbit Antibody Production and Purification

Both for comparison of immuno-affinity purification and for secondary antibody for ELISA and immunoblotting detection of β-glucosidase, rabbits were immunized with purified β-glucosidase as described in Materials and Methods. A HiTrap protein G Sepharose was used to purify polyclonal IgG from rabbit sera. Protein G, a cell surface protein of Group G streptococci is a type III Fc receptor that binds to the Fc region of IgG by non-immune mechanism.

High concentrations of immunoglobulin were obtained in 1 mL fractions collected during elution. Typically, each time antibody was purified, the highest concentration of immunoglobulin appeared in fractions 6 to 8. Fractions enriched in immunoglobulin were pooled and stored at -20 °C. After five consecutive purifications the amount of rabbit anti-β-glucosidase antibody obtained was 154 mg, representing an 88% recovery of IgY protein.

Purification and existence of polyclonal antibody was confirmed by SDS-PAGE shown in Figure 18. A single purified band of immunoglobulin with a molecular weight of approximately 150 kDa was detected (Figure 18-2). The HiTrap column was used to produce suitably purified antibody and its capability of

Figure 17.
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 7.5% gel) profile of duck anti-β-glucosidase antibody purified by Protein A Sepharose CL-4 B chromatography column.

Duck sera anti-β-glucosidase antibody (1) and duck anti-β-glucosidase purified by Protein A column chromatography (2). Each well contained 10 μg of protein.

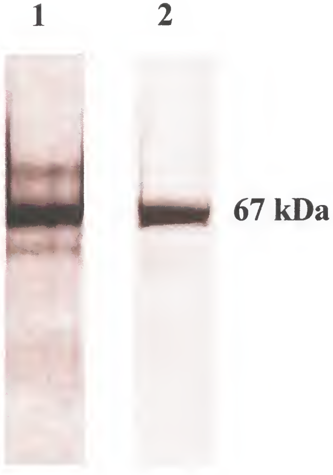
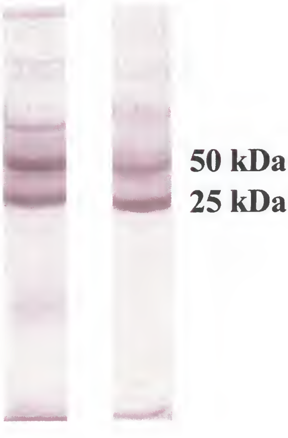

1
2
67 kDa

Figure 18.
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 7.5% gel) profile of rabbit anti-β-glucosidase antibody purified by HiTrap Protein G chromatography column.

Rabbit sera anti-β-glucosidase antibody (1) and rabbit anti-β-glucosidase purified by Protein A column (2). Each well contained 10 μg of protein.


50 kDa
25 kDa

quickly purifying Igs made it the preferred technique for isolation of immunoglobins.

Antibody Titer Determination

Antibodies from all three different sources showed affinity for β-glucosidase therefore, these antibodies may share recognition of some similar immunogenic determinants. Dose-related interactions of β-glucosidase at 2.5 to 95 ng of BG antigen with different sources of β-glucosidase specific antibodies at 75 ng each are shown in Figure 19. Of the three species duck, antibodies showed the highest sensitivity followed by rabbit and chicken, respectively. The appropriate dose range for the interaction of β-glucosidase with the different antibodies was determined to be 2.5 to 10 ng/well. When β-glucosidase was added at more than 10 ng/well, the antigen-antibody reaction was saturated and the sensitivity of the ELISA was reduced (Chen, 1991).

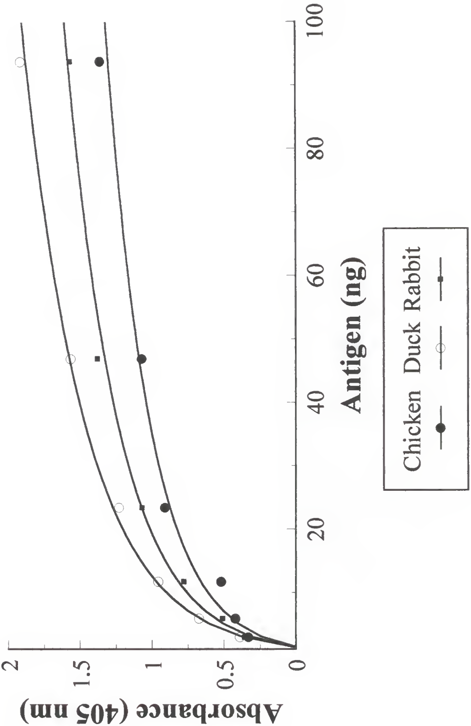
Hofer et al. (1989) reported that monoclonal antibody raised against β-glucosidase was able to detect as little as 10 ng of β-glucosidase. By comparison, results obtained in this research have yielded at least 4-times more sensitive polyclonal antibodies. This is an indication that avian species can be used for large quantity and high quality sources of antibodies.

Immunoblotting of Multiple Anti ***T. reesei*** β-Glucosidase Antibodies

In order to compare antibodies and evaluate their specificities toward β-glucosidase and other proteins an immunoblotting procedure was employed.

Figure 19.
Titer determination of antibodies (chicken, duck and rabbit) against β-glucosidase.

Chicken, duck and rabbit antibodies at 75 ng/well were used to react with purified β-glucosidase at 2.5 to 95 ng/well.
The result represents the average of eight replicates.

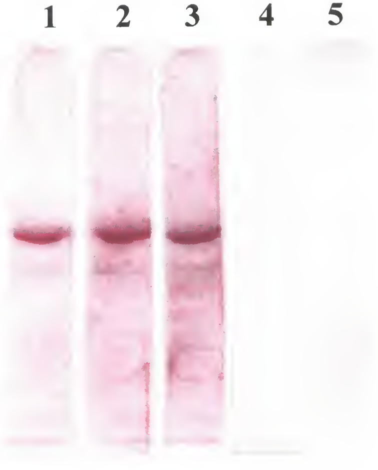

Absorbance (405 nm)
0
0.5
1
1.5
2
20
40
60
80
100
Antigen (ng)
Chicken
Duck
Rabbit

Purified β-glucosidase was electrophoresed through a 7.5% SDS-PAGE and transferred to a nitrocellulose membrane as described in Materials and Methods. Purified antibodies from the three species of animals were detected by reaction with electrophoretically separated β-glucosidase (Figure 20). An immune response was detected in chicken, duck and rabbit, respectively. A similar outcome was observed when the amount of β-glucosidase was lowered from 10 to 2 ng (data not shown). However, careful consideration has to be taken since overexposure of the cellulose membrane could yield false positive results. Therefore, the reaction should be stopped when antigen-antibody bands are evident.

Almond β-glucosidase, BSA as well as extra cellular cellulase-producing enzymes fungi were used as negative controls; a recognition of one of these proteins by the antibodies even at 1:100 lower sensitivity would invalidate their use. Ideally, other β-glucosidases from different ***Trichoderma sp.*** should be used as controls in order to determine specificity of the antibodies against the particular purified β-glucosidase of this report and investigate whether there is cross-reactivity. The lack of reactivity of each of the differing antibodies toward cellulases secreted by ***Hypocrea schweinitzii*** and ***Aspergillus niger*** once again confirmed the specificity of polyclonal antibodies from the different species for the enzyme and indicated that these antibodies might be an important tool in the development of new techniques such as purification of β-glucosidase by affinity chromatography.

Figure 20.
Specificity determination of anti-β-glucosidase antibodies by immunoblotting technique.

Chicken (1), duck (2) and rabbit (3) anti-β-glucosidase antibodies at 10 ng/mL were allowed to react against 10 ng of purified β-glucosidase. The rabbit and duck antibodies at 10 ng/mL were allowed to react with BSA (4) and ***H. schweinitzii*** cellulases (5).


1
2
3
4
5

Inhibitory Effect of Chicken Antibody on β-glucosidase

In order to decide which antibody was most appropriate for purification of β-glucosidase by affinity chromatography, inhibition of the antibodies on the enzyme was studied. Neither duck nor rabbit anti-β-glucosidase antibodies had a significant inhibitory effect on the purified enzyme. However, chicken anti-β-glucosidase antibody showed a strong inhibition of β-glucosidase.

The inhibition by chicken anti-β-glucosidase antibody of the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside catalyzed by purified β-glucosidase is illustrated in Figures 21 and 22. The Lineweaver-Burk and Dixon plot indicated that chicken anti-β-glucosidase antibody is a competitive inhibitor. A Dixon replot was constructed from the data presented in Figures 21 and 22 (Figure 23). A straight line through the origin indicated purely competitive inhibition. The inhibition constant (Ki) for the chicken anti-β-glucosidase antibody is 1.35 μg/mL, calculated both from replot of Dixon and Lineweaver-Burk plots, as well as by EZ-FIT software.

Egg yolk from hens contains at least two antigen-binding subclasses of IgY, derived from the hen serum and transmitted to the chick (Rose et al., 1974; Higgins, 1975). These immunoglobins due to polyclonal characteristics could recognize different epitopes of β-glucosidase and exert different modes of inhibition such as: (1) inhibitor and substrate are mutually exclusive because of steric hindrance; (2) inhibitor and substrate share a common binding group on the enzyme; (3) the binding sites for inhibitor and substrate are distinct but overlapping; or (4) the binding site of inhibitor to a distinct inhibitor site causes a conformational change in

Figure 21.
Lineweaver-Burk plot of β-glucosidase inhibition by chicken anti-β-glucosidase using *p*-nitrophenyl-β-D-glucopyranoside as substrate.

Reaction mixture contained 0.09 μg of purified β-glucosidase with *p*-nitrophenyl-β-D-glucopyranoside in 50mM sodium acetate buffer at pH 5.0. The fixed levels of inhibitor were 0 (•), 5 (◆), 10 (▲), 50 (■) and 100 (-) μg. The result represents the average of eight replicates.

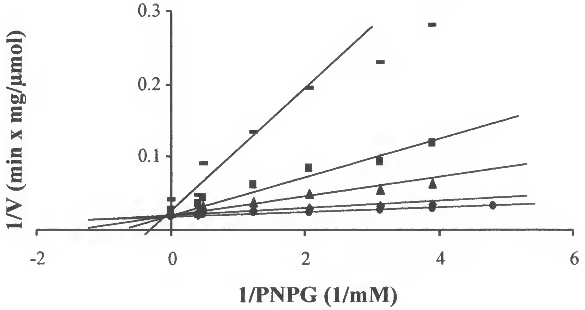

0.3
0.2
0.1
1/V (min x mg/µmol)
-2
0
2
4
6
1/PNPG (1/mM)

Figure 22.
Dixon plot of β-glucosidase inhibition by chicken anti-β-glucosidase antibody using *p*-nitrophenyl-β-D-glucopyranoside as substrate.

Substrate levels were 0.21 (■), 0.26 (+), 0.32 (◆), 0.48 (o), 0.80 (x), 2.00 (□) and 2.40 (•) mM.

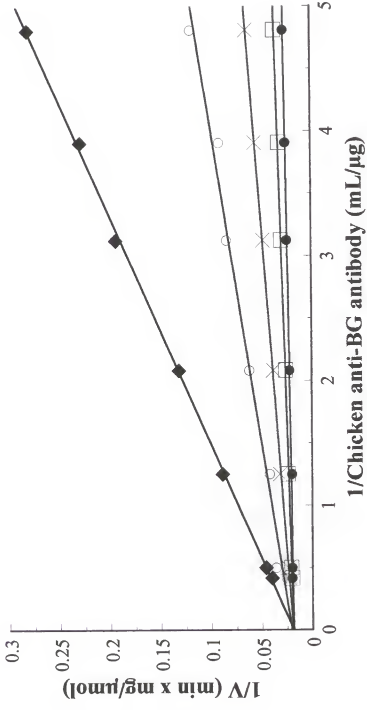
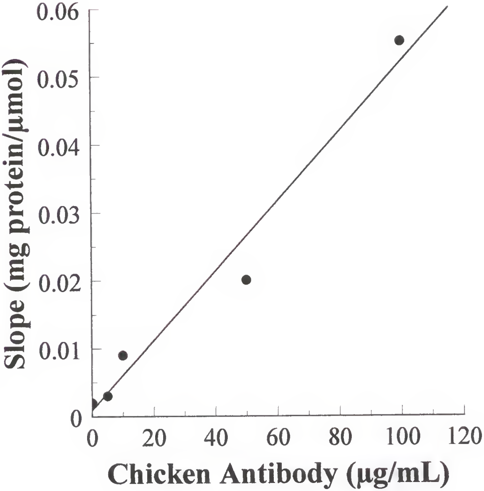

1/V (min x mg/µmol)
0.3
0.25
0.2
0.15
0.1
0.05
0
0
1
2
3
4
5
1/Chicken anti-BG antibody (mL/µg)

Figure 23.
Dixon replot of inhibition of β-glucosidase by chicken anti-β-glucosidase antibody.

p-nitrophenyl-β-D-glucopyranoside was used as substrate.


0.06
0.05
0.04
0.03
0.02
0.01
0
Slope (mg protein/μmol)
0
20
40
60
80
100
120
Chicken Antibody (μg/mL)

the enzyme that distorts or masks the substrate binding site and vice versa (Segel, 1976).

Furthermore, N-acetyl-glucosamine can be covalently linked to chicken antibodies as a post-translation modification (Preston, personal communication, 2000). Since glucose is *per se* an inhibitor of the β-glucosidase, a classical substrate and inhibitor competition for the same binding site can take place. A combination of all the above mentioned possibilities may explain the strong inhibition of this antibody against β-glucosidase.

Affinity Chromatography Purification of β-glucosidase

Since traditional purification required multiple steps and led to purified enzyme with minor contaminants, other more highly specific purification techniques were considered. Immobilized antibodies form different animal species were evaluated for these purposes. Several affinity matrices were used for the purification of β-glucosidase: cyanogen bromide-activated agarose, N-hydroxysuccinimidyl chloroformate activated agarose, and Affigel 10 and 15. The best result was obtained using N-hydroxysuccinimidyl chloroformate activated agarose, which for every 20 mg of covalently bound duck and rabbit antibodies, 0.40 mg of β-glucosidase was bound. The quantity of β-glucosidase bound was 0.31 or 0.18 mg (per 20 mg of bound duck or rabbit antibodies) for Affigel 15 or Affigel 10, respectively. Cyanogen bromide-activated agarose had the poorest yield of only 0.12 mg for either 20 mg of bound duck or rabbit antibodies linked to the gel. This difference may due to the fact that both rabbit and duck antibodies have acidic pIs, 6.1 (Fuller et al., 1997) and 5.4 (Higgins and Warr, 1995) respectively. No discernible difference was found with respect to the yield of matrices linked with duck or rabbit antibodies.

The efficiency of affinity purification of β-glucosidase by N-hydroxy-succinimidyl agarose chromatography is shown in Figure 24 and 25. Both immobilized rabbit and duck antibodies were able to purify β-glucosidase from different stages of purification by traditional isolation steps of the enzyme as starting material. Elimination of contaminants was evidenced by SDS-PAGE whereas after each purification a single band was observed with the sole exception when culture filtrate was used as starting material (data not shown). This suggests that first an anion exchange chromatographic step is needed in order to remove some of the contaminants in the solution. However, eliminating some of the steps of traditional purification saves both time and material. The specific activity of the purified enzyme by affinity chromatography was calculated to be 43.5 units/mg; the same values of Km (0.14 mM) and Vmax (53.9 units/mg) were obtained. These results are similar to those achieved by the traditional purification. Tomaz and Queiroz (1999) separated β-glucosidase from ***Trichoderma reesei*** using a combination of gel permeation and hydrophobic interaction chromatography. They obtained only a two-fold increase in specific activity. Therefore, although they used only two purification steps, the specific activity obtained of the enzyme was only 22% of that by affinity purification.

Figure 24.
Sodium dodecyl sulfate polyacrylamide electrophoresis
(SDS-PAGE, 7.5% gel) gel profile of β-glucosidase purified by
duck antibody hydroxysucciminidyl-agarose (NHS) affinity chromatography column.

β-glucosidase eluted from DEAE Sephadex succinate buffer column (A),
purified β-glucosidase eluted from NHS column using sample A as starting material (A*).
β-glucosidase eluted from DEAE Sephadex imidazole buffer column (B),
purified β-glucosidase eluted from NHS column using sample B as starting material (B*).
β-glucosidase eluted from SP-Sephadex column (C),
purified β-glucosidase eluted from NHS column using sample C as starting material (C*)
Each well contained 5 μg of protein.

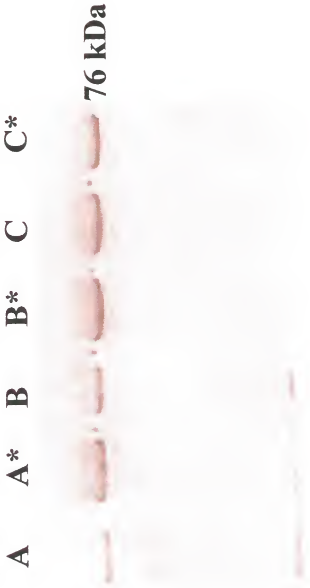
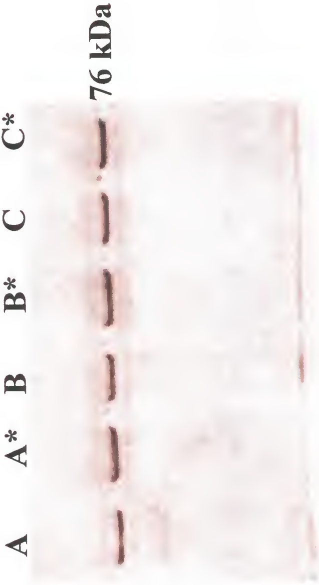

A
A*
B
B*
C
C*
76 kDa

Figure 25.
Sodium dodecyl sulfate polyacrylamide gel electrophoresis
(SDS-PAGE, 7.5% gel) profile of β-glucosidase purified by
rabbit antibody hydroxysucciminidyl-agarose affinity chromatography column.

β-glucosidase eluted from DEAE Sephadex succinate buffer column (A),
purified β-glucosidase eluted from NHS column using sample A as starting material (A*).
β-glucosidase eluted from DEAE Sephadex imidazole buffer column (B),
purified β-glucosidase eluted from NHS column using sample B as starting material (B*).
β-glucosidase eluted from SP-Sephadex column (C),
purified β-glucosidase eluted from NHS column using sample C as starting material (C*)
Each well contained 5 μg of protein.


A
A*
B
B*
C
C*
76 kDa

SUMMARY AND CONCLUSIONS

Purification of an extracellular β-glucosidase from a strain of ***Trichoderma reesei*** containing multiple *bgl1* genes has been achieved through the use of a combination of traditional ion exchange chromatography and by a novel affinity approach. The enzyme was characterized with respect to enzymatic properties. By traditional methods, purification was accomplished through the use of two successive anion exchange chromatography steps, followed by cation exchange chromatography. Purified enzyme was also obtained by affinity chromatography using anti-β-glucosidase antibodies raised in duck and rabbit. Evidence of homogeneity is provided by SDS-PAGE patterns of the purified enzyme which show a single protein band.

Molecular weight analysis indicated a 76000 Da protein. A single band was obtained after SDS-PAGE indicating that the purified enzyme is composed of a single, monomeric polypeptide. The purified β-glucosidase is rich in basic amino acids and contains few half cystine and methionine residues. This protein is isoelectric at pH 8.4, clearly distinguishing it from both the extracellular β-glucosidase of ***Trichoderma viride*** and the intracellular β-glucosidase from a native strain of ***Trichoderma reesei***, both of which are more acidic. The high isoelectric point suggests an ionic attraction between the basic β-glucosidase and acidic depolymerizing enzymes of the cellulase system. The lack of affinity of this enzyme for Concanavalin A indicated that the β-glucosidase contains little or no α-D-mannopyranosyl and/or α-D-glucopyranosyl terminal groups or internal 2-*O*-D-mannopyranosyl residues. The amino acid compositions of the purified β-glucosidase of the mutant strain and that isolated from native strain are similar.

The lability of the β-glucosidase at low pH distinguishes it from the extracellular β-glucosidase from ***Trichoderma viride***. Log Vmax and Log Vmax/Km versus pH profiles were similar and each was dependent on group(s) having pKa of 5.5 and 7.3, respectively. The enzyme is optimally active from pH 4.5 to 5.0 and loses its activity at lower pHs.

The values of Km and Vmax for *p*-nitrophenyl-β-D-glucopyranoside as substrate were determined to be 0.14 mM and 54 units/mg, respectively. The β-glucosidase had an unusually high affinity for D-glucose and was strongly inhibited by 1-deoxynojirimycin, whereas the inhibition constants were calculated to be 638 μM and 0.62 μM respectively. Both inhibitors acted in a competitive mode. Inhibition by 1-deoxynojirimycin is due to its resemblance to the glucopyranosyl cation, which is considered to be the transition state formed during β-glucosidase-catalyzed hydrolysis of glucopyranosides.

The purified β-glucosidase of the transformed strain exhibits the same characteristics (MW, pI, pH optimum, Ki, Km and Vmax) as the native strain. Through genetic transformation an increased yield of extracellular β-glucosidase has been attained; this strain produces a level of extracellular β-glucosidase some 50-fold greater than the parent strain and could be used for different commercial purposes.

Avian species are inexpensive and easy to handle and a convenient source of polyclonal antibodies, since the quantity of antigen needed for an efficient immune response is much lower than in rabbits. The use of complete Freund's adjuvant leads to very high and long-lasting titers of antibodies starting as early as 10 or 14 days after the first immunization for duck and chicken, respectively. Egg collection is an easy and non-invasive method to obtain antibodies from birds; the yolk may contain more than 45 mg of immunoglobulins per unit.

The purification of antibodies was fast and simple. Both methods used in this report to isolate the chicken antibodies yielded a high quantity of immunoglobulins, although the procedure described by Hassl and Aspöck (1988) is more rapid than the PEG precipitation described by Polson et al. (1985). For use in affinity chromatography antibody purification utilized either protein A (for duck) or HiTrap protein G (for rabbit) and yielded a 92% recovery and a total of 109 mg of duck antibodies and 154 mg of rabbit antibodies representing 88% recovery. Purification was confirmed by SDS-PAGE which yielded a single band for each of the three species of anti-β-glucosidase antibodies. These results established the efficiency of these purification methods.

β-Glucosidase antibody sensitivity and specificity were determined by the methods of ELISA and immunoblotting, respectively. The results indicated that as little as 2.5 ng of antibodies were capable of detecting 10 ng of purified β-glucosidase; this is at least a four-fold higher sensitivity than that previously reported for monoclonal antibody raised against the same β-glucosidase. An immunoblotting study showed the specificity of the antibody for the enzyme, by yielding a single band on nitrocellulose membrane after Western blotting. Each of the three species of antibodies was able to detect as little as 2 ng of purified β-glucosidase. Nonetheless, chicken anti-β-glucosidase antibodies were found to bind poorly to immobilized protein A and protein G, also showed a strong, competitive inhibition effect on the enzyme, for which Ki was calculated to be 1.36 μg/mL. This phenomenon may be due to the presence of more than two subclasses of antigen binding IgY in egg yolk. These subclasses of chicken antibodies could interact with the antigen in some distinctly unique way or as a combination of different modes of competitive inhibition that reduce the activity of the enzyme. Furthermore, N-acetyl-glucosamine residues in chicken immunoglobulins might be responsible for the inhibition of β-glucosidase; these residues might lead to inactivation or association with the active site of the enzyme.

Affinity purification by N-hydroxysuccinimidyl chloroformate-activated agarose chromatography using immobilized duck and rabbit anti-β-glucosidase antibodies proved to be a superior tool for the purification of β-glucosidase. For every 20 mg of duck and rabbit antibodies, 0.4 mg of β-glucosidase was bound. The resulting elimination of contaminants and reduction of traditional purification steps were demonstrated by SDS-PAGE. A single band of β-glucosidase was observed after affinity chromatography using different stages of purification of β-glucosidase as starting material, achieving the same specific activity value of 43.5 units/mg and the same Km (0.14 mM) and Vmax (53.9 units/mg) as the traditional ionic exchange chromatography purification procedure. Now that the affinity of the immobilized antibodies for β-glucosidase has been established, some chromatographic procedures may be eliminated (DEAE Sephadex A-50, 5 mM imidazole buffer and SP Sephadex C-50, 20 mM succinate buffer) and the purification of the enzyme can be performed more rapidly.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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